Signal-regulatory protein α-mediated inhibition of monocyte adhesion to arterial elastic laminae

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ABSTRACT

Elastic laminae are extracellular matrix constituents that not only determine the stability and elasticity of arteries, but also play a role in regulating arterial morphogenesis and pathogenesis. We demonstrate here that an important function of elastic laminae is to prevent leukocyte activation and inflammatory reactions. In an arterial replacement model in vivo based on decellularized matrix scaffolds, elastic laminae are resistant to leukocyte adhesion and transmigration compared with collagen-rich adventitia. The density of leukocytes within the elastic laminae was about 58 to 70-fold lower than that within the adventitia from 6 hrs to 30 days after surgery. Elastic lamina degradation peptides (ELDPs) extracted from arterial specimens bind to and activate the inhibitory receptor signal-regulatory protein (SIRP) α in monocytes, and induce the recruitment and phosphorylation of SH2 domain-containing protein tyrosine phosphatase (SHP)-1, leading to reduced monocyte adhesion and release of monocyte chemotactic protein (MCP)-1 in vitro. The blockage of SIRP α with an anti-SIRP α antibody reduced the anti-inflammatory effects of the elastic laminae. These observations suggest that SIRP α potentially mediates the anti-inflammatory effects of arterial elastic laminae.

INTRODUCTION

Vascular elastic laminae have long been considered structures that determine the strength and elasticity of blood vessels[1-5]. Recent studies have demonstrated that arterial elastic laminae play a role in the regulation of arterial morphogenesis and pathogenesis[6-8]. An important contribution of elastic laminae is to confine smooth muscle cells (SMCs) to the arterial media by inhibiting SMC proliferation[7,9] and migration[8], thus preventing intimal hyperplasia under physiological conditions. Arterial elastic laminae also exhibit thrombosis-resistant properties. In a matrix implantation model in vivo, an elastic lamina scaffold is associated with significantly lower leukocyte adhesion, thrombosis, and intimal hyperplasia compared with collagen-rich adventitia[8]. These observations suggest an anti-inflammatory role for elastic laminae. While such a role is well documented, the mechanisms remain poorly understood.

Leukocytes are known to express the inhibitory receptor SIRP α (also known as Src homology 2 domain-containing tyrosine phosphatase substrate-1), a glycoprotein receptor that exerts inhibitory effects on cell mitogenic[10-15] and inflammatory activities[16,17]. SIRP α is a transmembrane receptor that transmits inhibitory signals through tyrosine phosphorylation of its intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM)[12–15,18,19]. The phosphorylation of the ITIM, upon ligand binding to SIRP α, initiates the recruitment of Src homology 2 domain-containing tyrosine phosphatase (SHP)-1 to SIRP α, which is known as a substrate of SHP-1[18,19]. The recruitment of SHP-1 also localizes and activates SHP-1[20], which in turn dephosphorylates protein

kinases, possibly including receptor tyrosine kinases[20-22], Src family protein tyrosine kinases[23], phosphatidylinositol 3-kinase[23], and the Janus family tyrosine kinases[24,25]. These activities potentially suppress inflammatory and mitogenic responses[26-29]. On the basis of these previous investigations, it is hypothetically possible that, upon leukocyte contact, elastic laminae may interact with and activate SIRP α, which in turn inhibits leukocyte adhesion. In this study, we provide experimental evidence for such a hypothesis.

METHODS

Preparation of aortic matrix scaffolds. Rats (Sprague Dawley, male, 300-350 gm) were anesthetized with intraperitoneal injection of 50 mg/kg sodium pentobarbital. The aorta was removed, turned inside out to expose the endothelial surface, incubated in 0.1 M NaOH at 23° C for 2 hrs with vigorous agitation, and washed in a large volume of PBS for 12 hrs with vigorous agitation. Such a treatment removes the cellular components, basal lamina, and medial collagen matrix, leaving an aortic matrix scaffold with medial elastic laminae and collagen-dominant adventitia[8].

Arterial replacement. A host rat was anesthetized as described above. Allogenic aortic matrix scaffolds with two types of blood-contacting surface, elastic lamina and adventitia (generated by turning the matrix scaffold outside in), were prepared as described above and used for arterial replacement. A host jugular vein was harvested and used as a control. The two matrix scaffolds and the control jugular vein were anastomosed into the host aorta in a series in the order of adventitia, jugular vein, and elastic lamina by using a method described previously[30]. Observations were carried out at 0.25, 1, 5, 10, 20, and 30 days with 4 rats at each observation time. Experimental procedures were approved by the Animal Care and Use Committee of Northwestern University.

Measurement of leukocyte adhesion and transmigration in vivo. At each observation time, a rat was anesthetized as described above. To measure leukocyte adhesion to the surface of matrix scaffolds, specimens were collected from all three types of arterial replacement, fixed in 4% formaldehyde in PBS, incubated with

phycoerythrin-conjugated anti-CD 11 b/c antibody (Caltag) and Hoechst 33258, and observed en face by fluorescence microscopy. The density of CD 11 b/c-positive cells on the surface of each specimen was measured and compared between different specimens. To assess leukocyte transmigration, fixed specimens were cut into transverse cryo-sections of 10 μm in thickness, and processed as described above for measuring the density of CD11 b/c-positive cells within the elastic lamina-dominant media and collagen-dominant adventitia. Results were compared between the two layers.

Measurement of neointimal formation. Specimens were collected from aortic matrix scaffolds and control vein grafts, fixed in 4% formaldehyde, and cut into transverse cryo-sections of 10 μm in thickness. The specimen sections were incubated with an anti-SMC α actin antibody (Chemicon) at 37° C for 1 hr, incubated subsequently with a phycoerythrin-conjugated secondary antibody, and used for measuring the thickness of SMC α actin-containing layers by fluorescence microscopy.

Preparation of monocytes. Rats were anesthetized as described above. A blood sample of approximately 12 ml was collected from the vena cava of each rat, mixed with 20% acid-citrate-dextrose (120 mmol/L sodium citrate, 110 mmol/L glucose, and 80mmoi/L citric acid). Monocytes were collected by using a monocyte enrichment kit (Stemcell Technologies, Vancouver) according to the manufacturer's instructions. Enriched monocytes were suspended in host plasma at a cell count ~6 x 10^6 cells/ml, supplemented with 100 μg/ml streptomycin and 100 U/ml penicillin.

Monocytes were identified with an anti-CD 11 b/c antibody (Caltag). The majority of enriched cells were CD 11 b/c-positive.

Measurement of monocyte adhesion in vitro. Enriched monocytes were incubated in host plasma at 37^{o} C in the presence of elastic lamina and adventitial specimens for 3, 6, 12, and 24 hrs with gentle agitation. At each time, a segment was removed from each type of matrix specimen, fixed in 4% formaldehyde in PBS, incubated with Hoechst 33258, and observed with a fluorescence microscope for measuring the density of monocytes adhered to the matrix specimens. To test the influence of protein tyrosine phosphatases on monocyte adhesion to elastic laminae, a protein tyrosine phosphatase inhibitor, sodium orthovanadate[31], was applied to monocyte assays and the density of monocytes on elastic lamina specimens was measured in the presence and absence of sodium orthovanadate (10 μm).

Measurement of monocyte release of MCP-1. Rat monocytes were incubated in host plasma to assess the influence of rat arterial elastic lamina degradation peptides (ELDPs) on monocyte release of MCP-1. ELDPs were prepared by KOH digestion as described previously[32] and applied to cultured monocytes at a concentration of 10 μg/ml. Since quiescent monocytes release a relatively low level of MCP-1, monocytes were incubated with 100 nM phorbol myristate acetate (PMA) for 30 min to stimulate MCP-1 release, followed by incubation with and without 10 μg/ml ELDPs. At incubation times 0.5, 1, 3, and 6 hrs, monocytes were removed via centrifugation, and the supernatants (0.5 ml at each time) were mixed with protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM PMSF, and 1 mM benzamidine) and processed for detecting the relative level of MCP-1 by

immunoprecipitation and immunoblotting. Briefly, the supernatants were measured for total protein concentrations, precleaned with protein A-conjugated agarose beads (10% packed beads, Upstate), immunoprecipitated with an anti-MCP-1 antibody (4 μg/ml, Chemicon) at 4° C for 4 hrs, and incubated with protein A-agarose beads (10% packed beads) at 4° C for 4 hrs. The agarose beads were collected and treated with protein sample buffer at ~100° C for 5 min. Immunoprecipitates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the same antibody (0.5 μg/ml). Relative protein signals were examined by secondary peroxidase-IgG labeling and chemiluminescent detection of peroxidase activities[33].

Assessment of ELDP binding to SIRP α. To detect whether ELDPs bind to SIRP α, monocytes were cultured in the presence of 10 μg/ml ELDPs, collected at 0.5, 1, and 3 hrs, and lysed in lysis buffer, containing 1% Triton X-100, 150 mM NaCI, 10 mM Tris-HCI (pH 7.4), 2 mM sodium orthovanadate, 0.4 mM EDTA, and a protease inhibitor cocktail (1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM PMSF, and 1 mM benzamidine). Lysates were processed for immunoprecipitation with an anti-elastin antibody (Elastin Products), resolved by SDS-PAGE, and probed with an anti-SIRP α antibody (Santa Cruz) and an anti-phosphotyrosine antibody (4G10, Upstate) as described above.

Detection of SIRP α and SHP-1 phosphorylation. To detect whether ELDP binding induces SIRP α phosphorylation and whether SIRP α phosphorylation induces SHP-1 recruitment, monocytes were cultured in the presence and absence of ELDPs (10 μg/ml), collected at 0.5, 1, 3, and 6 hrs, and lysed in lysis buffer.

Lysates were processed for immunoprecipitation with an anti-SIRP α antibody (Santa Cruz) and for sequential immunoblotting by using anti-SIRP α, anti-SHP-1 (Santa Cruz), and anti-phosphotyrosine (4G10, Upstate) antibodies with antibody stripping after each immunoblotting reaction. The relative expression and tyrosine phosphorylation of SIRP α and SHP-1 were detected by using a chemiluminescent method[33].

Detection of ELDP binding to monocytes. To test whether ELDPs bind to monocytes, freshly collected monocytes were cultured at 37° C for 1 hr in the presence of 10 μg/ml fluorescein-conjugated ELDPs, prepared by using a fluorescein-labeling kit (Roche) according to the manufacturer's instructions. Monocytes incubated with an unrelated fluorescein-conjugated secondary antibody (10 μg/ml) were used as a control. Monocytes were washed in PBS and observed by fluorescence microscopy.

Cytometry confirmation of ELDP binding to SIRP α. To confirm the binding of ELDPs to SIRP α, the influence of an anti-SIRP α antibody (Santa Cruz), developed with the extracellular domain (1 - 300 amino acids) of SIRP α as an antigen and known to bind to SIRP α, on the relative binding of ELDPs was detected in a competition assay by flow cytometry. Monocytes were treated with 0, 5 and 10 μg/ml anti-SIRP α antibody separately at 37° C for 1 hr, and subsequently incubated with 10 μg/ml fluorescein-conjugated ELDPs at 37° C for 1 hr. Monocytes incubated with an unrelated fluorescein-conjugated secondary antibody (5 and 10 μg/ml) were used

as control for the competition assay. Monocytes were detected for fluorescent intensity by flow cytometry (Beckman Coulter Epics XL-MCL).

Influence of anti-SIRP α antibody on the anti-inflammatory effects of ELDPs and elastic laminae. To confirm whether SIRP α mediates the anti-inflammatory effects of ELDPs, an anti-SIRP α antibody (developed with the extracellular domain of SIRP α 1-300 amino acids as an antigen by Santa Cruz) was used to assess the influence of SIRP α on monocyte release of MCP-1 in the presence and absence of ELDPs. Monocytes were first incubated with PMA (100 nM) for 30 min to stimulate MCP-1 release, incubated with the anti-SIRP α antibody (10 μg/ml) for 1 hr, and subsequently incubated with ELDPs (10 μg/ml) for 1 and 3 hrs. Monocytes incubated with the anti-SIRP α antibody alone (10 μg/ml, without ELDPs) and monocytes alone (without anti-SIRP α antibody and without ELDPs) were used as controls. An unrelated secondary antibody (10 μg/ml) was used as a control for the blocking assay. The relative level of MCP-1 in the culture plasma was assessed as described above. In addition, monocytes were incubated with elastic lamina and adventitial specimens in the presence and absence of the anti-SIRP α antibody (10 μg/ml). The influence of the anti-SIRP α antibody on monocyte adhesion to elastic laminae and adventitia was assessed at 1 and 3 hrs.

Statistical Analyses. Means and standard deviations were calculated for each measured parameter at each observation time. The Student t-test was used for difference comparison between two groups. A difference is considered statistically significant at $p < 0.05$.

RESULTS

Elastic laminae are resistant to leukocyte adhesion and transmigration

To demonstrate the anti-inflammatory role of arterial elastic laminae, we prepared decellularized matrix scaffolds from the rat aortae, replaced a segment of the host rat aorta by using matrix scaffolds with two different blood-contacting surfaces: elastic lamina and collagen-dominant adventitia. The influence of elastic lamina and collagen matrix on leukocyte adhesion and transmigration was examined at selected times. As shown in Fig. 1A, almost all cells found in the aortic matrix scaffold were CD11 b/c-positive leukocytes (predominantly monocytes/macrophages and granulocytes) within the first 5 days. While massive leukocytes migrated into the collagen-dominant adventitia, few leukocytes were found within the elastic lamina-dominant media. The density of leukocytes in the media was 58- to 70-fold lower than that in the adventitia from 6 hrs to 30 days after surgery (Fig. 1B). At sites of ruptured elastic laminae, leukocytes were not able to migrate into the gaps between the elastic laminae, even though the gaps are apparently larger than the diameter of leukocytes (Fig. 1A 10 days*). On the blood-contacting surface, the degree of leukocyte adhesion to the elastic lamina was significantly lower than that to the adventitia (Fig. 1C). Furthermore, the elastic lamina scaffold was associated with significantly thinner thrombus/neointima than the adventitia (Fig. 2). These observations suggest that, compared with collagen matrix, elastic laminae are resistant to leukocyte adhesion and transmigration.

To further confirm the anti-inflammatory effects of arterial elastic laminae, we collected monocytes from rat blood, incubated these cells with specimens of decellularized elastic laminae and collagen-dominant adventitia from the rat

aorta, and examined monocyte adhesion to these matrix specimens in vitro. As shown in Fig. 3, the exposure of monocytes to adventitia induced monocyte adhesion ranging from 149 to 268 cells/mm^2 within 24 hrs. In contrast, exposure to elastic laminae resulted in about 18-fold lower monocyte adhesion (8 to 15 cells/mm^2 within 24 hrs).

ELDPs inhibit monocyte release of MCP-1

Monocytes express and release MCP-1, which potentially enhances leukocyte adhesion and activation. To explore whether MCP-1 is involved in elastic lamina-mediated monocyte activities, we extracted ELDPs from rat aortae by KOH digestion, and assessed the influence of ELDPs on monocyte release of MCP-1 in vitro. As shown in Fig. 4A, a treatment with ELDPs (10 μg/ml) reduced PMA-stimulated release of MCP-1 in monocytes. These observations support the anti-inflammatory role of arterial elastic laminae.

Sodium orthovanadate enhances monocyte adhesion and MCP-1 release

Protein tyrosine phosphatases are known to inhibit inflammatory activities. It is possible that the protein tyrosine phosphatases may mediate the anti-inflammatory effects of elastic laminae. To test such a possibility, we examined the influence of sodium orthovanadate, a protein tyrosine phosphatase inhibitor, on monocyte adhesion to elastic laminae and monocyte release of MCP-1. As shown in Fig. 3A and B, a treatment with sodium orthovanadate (10 μM) induced a significant increase in monocyte adhesion to elastic laminae. Sodium orthovanadate also caused an apparent increase in monocyte release of MCP-1 in the presence of ELDPs (Fig. 4B). These observations support the role of

protein tyrosine phosphatases in mediating the anti-inflammatory effects of arterial elastic laminae.

ELDPs bind to SIRP α

To test the possibility that SIRP α may serve as a mediator for the anti-inflammatory effects of elastic laminae, we examined whether arterial ELDPs bind to SIRP α in monocytes by immunoprecipitation and ligand competition assays. As shown in Fig. 5A, in monocytes incubated with ELDPs (10 μg/ml) in vitro, SIRP α could be co-immunoprecipitated with ELDPs by using an anti-elastin antibody. Furthermore, fluorescein-conjugated ELDPs (10 μg/ml) were capable of binding to monocytes (Fig. 5B). A treatment with an anti-SIRP α antibody (5 and 10 μg/ml), developed with the extracellular domain (1 – 300 amino acids) of SIRP α as an antigen, competitively reduced the binding of fluorescein-conjugated ELDPs (10 μg/ml) to monocytes (Fig. 5C). These observations suggest that ELDPs bind to SIRP α in rat monocytes.

ELDPs activate SIRP α and induce the recruitment and phosphorylation of SHP-1

Tyrosine phosphorylation of SIRP α is required for its anti-inflammatory effects and for the recruitment and activation of SHP-1. To test whether the binding of ELDPs to SIRP α induces SIRP α phosphorylation and SHP-1 recruitment, we examined co-immunoprecipitation of SIRP α with SHP-1 and the relative phosphorylation of these molecules in the presence of ELDPs. As shown in Fig. 6A, a treatment with ELDPs (10 μg/ml) induced SIRP α phosphorylation,

and heavily phosphorylated SIRP α was associated with increased co-immunoprecipitation with SHP-1 in monocytes, suggesting that SIRP α phosphorylation enhanced SHP-1 recruitment. The recruitment of SHP-1 was associated with an apparent increase in SHP-1 phosphorylation in the presence of ELDPs (Fig. 6A). These observations suggest that the binding of ELDPs to SIRP α activates SIRP α and induces the recruitment and phosphorylation of SHP-1.

Blockage of SIRP α reduces the anti-inflammatory effects of ELDPs and elastic laminae

To confirm the role of SIRP α in mediating the anti-inflammatory effects of ELDPs, we applied an anti-SIRP α antibody, developed with the extracellular domain of SIRP α (1-300 amino acids) as an antigen, to cultured monocytes to block ligand binding to SIRP α. A pretreatment with the anti-SIRP α antibody (10 μg/ml for 1 hr) diminished the inhibitory effects of ELDPs on monocyte release of MCP-1 (Fig. 6B), and induced a significant increase in monocyte adhesion to elastic laminae (Fig. 6C), while it did not significantly influence monocyte adhesion to adventitia (Fig. 6D). These observations support the role of SIRP α in mediating the anti-inflammatory effects of elastic laminae.

DISCUSSION

The anti-inflammatory effects of elastic laminae

Biological activities are often controlled by coordinated activating and inhibitory signaling mechanisms, so that the activities can be initiated and terminated promptly and selectively. For instance, protein phosphorylation and dephosphorylation are typical opposing signaling processes that promote and inhibit cellular activities, respectively, for certain mitogenic signaling pathways. Extracellular matrix components participate in the regulation of cellular activities, such as cell adhesion, proliferation, migration, and pattern formation. Collagen-containing matrices have long been known to stimulate these cellular processes[34,35]. However, the inhibitory aspect of extracellular matrix has been poorly understood. Several recent studies have suggested a role for elastic laminae in regulating the proliferation and migration of vascular SMCs. Genetically induced deficiency of elastin in a mouse model causes enhanced SMC proliferation, intimal hyperplasia, and arterial stenosis during the fetal stage, resulting in animal death early after birth[8]. Incomplete development of arterial elastic laminae in human genetic disorders, such as Williams syndrome and supravalvular aortic stenosis, is associated with similar pathological changes in large arteries[36-38]. In a model of arterial implantation, decellularized elastic laminae prevent SMC migration into the elastic laminae[8]. These observations have demonstrated that elastic laminae exert inhibitory effects on mitogenic activities of vascular SMCs. Such inhibitory effects may counterbalance the stimulatory effects of collagen matrices.

The present study demonstrates that arterial elastic laminae are resistant to leukocyte adhesion and transmigration compared with collage-rich adventitia. It is interesting to note that the density of leukocytes within the elastic laminae was 58 to 70-fold lower than that within the collagen-dominant adventitia. These observations suggest an anti-inflammatory role for the arterial elastic laminae. The inhibitory effects of elastic laminae on leukocyte activities were associated with a reduction in intima-medial hyperplasia compared with autogenous vein grafts and collagen scaffolds. Such inhibitory effects may contribute to the relatively low-level inflammatory reactions in the arterial media compared with that in the intima as seen in arteries with injured endothelium.

The discovery of the anti-inflammatory effects of arterial elastic laminae suggests that elastic laminae may be potentially used for constructing the blood-contacting surface of arterial substitutes. During the past decades, investigators have been searching for an ideal arterial substitute that possesses the structural and mechanical features of a natural artery and antithrombotic properties. A number of polymeric and biological materials, including non-biodegradable polymers[39], biodegradable polymers[40–44], collagen matrix[45–47], and fibrin matrix[48], have been characterized and tested in clinical or experimental studies. While each of these biomaterials exhibits characteristics suitable for the construction of arterial substitutes, the patency of biomaterial-based arterial substitutes remains problematic because of inflammation and thrombogenesis[49]. Endothelial cell seeding of biomaterials has been proposed and used for reducing the thrombogenicity of biomaterials[50]. However, difficulties in cell retention during and after arterial reconstruction hinder the application of such an approach[51]. Although

autogenous vein grafts offer satisfactory results[30,52], not all patients possess veins available for arterial reconstruction. Thus a pressing issue in arterial reconstruction is to develop arterial substitutes that are inflammation/thrombosis-resistant and possess the structural and mechanical properties of natural arteries. Given the natural features, anti-inflammatory and antithrombotic properties, inert immunogenicity, and availability of allogenic arterial elastic laminae, these laminae may be used potentially for arterial reconstruction.

Potential mechanisms of the anti-inflammatory effects of elastic laminae

Inflammation is a critical process that contributes to thrombogenesis and atherogenesis. Two opposing mechanisms, protein tyrosine kinase-induced phosphorylation and protein tyrosine phosphatase-induced dephosphorylation, are potentially involved in the regulation of inflammatory activities[26-29]. While the activation of certain protein tyrosine kinases may initiate and promote inflammatory activities, the activation of corresponding protein tyrosine phosphatases may exert opposite effects. Myeloid cells, including monocytes, express the inhibitory receptor SIRP α[10-12]. The activation of such a receptor may recruit and activate SHP-1[18-20]. Activated SHP-1 in turn dephosphorylates or deactivates substrate protein tyrosine kinases, thus inhibiting inflammatory activities[20-29]. In motheaten mice with SHP-1 deficiency, profound activation of macrophages and neutrophils occurs in conjunction with inflammatory reactions in the lung, liver, joints, and dermal tissue[53]. These observations confirm the role of SHP-1 in suppressing inflammatory activities. Thus, the SIRP α - SHP-1 signaling pathway provides a

potential system that may mediate the anti-inflammatory effects of arterial elastic laminae.

The present study demonstrates that elastic lamina degradation peptides (ELDPs) are capable of binding to and activating SIRP α, which subsequently recruits and activates SHP-1, in rat monocytes. The activation of SIRP α was consistent with the reduction of monocyte adhesion and MCP-1 release induced by exposure to elastic laminae or ELDPs, suggesting a potential role for SIRP α in mediating the anti-inflammatory effects of elastic laminae. Several observations support the involvement of SIRP α. First, ELDPs are capable of binding to and activating SIRP α, as shown by immunoprecipitation and competitive ligand-binding assays. Second, the blockage of SIRP α with an anti-SIRP α antibody reduced the anti-inflammatory effects of elastic laminae or ELDPs.

Elastic laminae may interact with not only SIRP α, but also other types of receptor, including the laminin/elastin receptor[1,3,54]. Interestingly, the binding of elastin to the laminin/elastin receptor has been reported to activate mitogenic signaling mechanisms and promote the proliferation of cultured SMCs[54], although different phenomena have been observed[7,55]. These observations, together with the information addressing the inhibitory aspect of elastic laminae, suggest that activating and inhibiting receptors may coexist and coordinate in the regulation of leukocyte adhesion and transmigration. The final consequence may depend on the relative strength of the activating and inhibiting receptors.

In conclusion, the present observations suggest that, compared with collagen matrix, arterial elastic laminae and ELDPs suppress leukocyte adhesion and transmigration, and exert anti-inflammatory effects. These effects are potentially mediated by the inhibitory receptor SIRP α. The interaction of elastic laminae with leukocyte SIRP α may activate signaling pathways that potentially inhibit adhesion-stimulating mechanisms. The inhibitory effects of elastic laminae potentially counterbalance the stimulatory effects of collagen matrix, contributing to coordinated regulation of leukocyte transmigration. Furthermore, the anti-inflammatory features render elastic lamina a potential blood-contacting material for arterial replacement, an effective approach for the treatment of atherosclerosis.

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Figure Legends

Figure 1. Leukocyte adhesion and migration to elastic lamina-dominant media and collagen-dominant adventitia of aortic matrix scaffolds. (**A**) Transverse fluorescent micrographs showing the migration of CD 11b/c-positive leukocytes into the adventitia (a), but not into the elastic laminae of matrix scaffolds (e). Note that leukocytes did not migrate into the gaps between the elastic laminae at the end or rupture sites of the matrix scaffold (indicated by * in panel 10 days*). Red: antibody-labeled CD 11 b/c. Blue: Hoechst 33258-labeled cell nuclei. Green: elastic laminae. Arrow: blood-contacting surface. Scale: 100 μm. (**B**) Measurements of cell density in the elastic lamina-dominated media and collagen-dominated adventitia of matrix scaffolds. Differences were significant ($p < 0.0001$) at all observation times except time 0, at which no cells were present in the media and adventitia (removed by NaOH treatment). Means and standard deviations are presented. (**C**) Measurements of leukocyte adhesion to the elastic lamina scaffold, adventitial scaffold, and autogenous vein graft. Differences were significant between elastic lamina and adventitial scaffolds and between vein grafts and adventitial scaffolds at all observation times ($p < 0.001$), but were not significant between elastic lamina scaffolds and vein grafts at all observation times ($p > 0.05$). Means and standard deviations are presented.

Figure 2. Neointimal formation in elastic lamina and adventitial scaffolds. (**A**) Transverse fluorescent micrographs showing anti-SMC α actin antibody-labeled cells (red) and elastic laminae (green). Arrow: blood-contacting surface. a: adventitia. e: elastic laminae. Scale: 100 μm. (**B**) Measurements of the thickness of SMC α actin-containing intima-media in matrix scaffolds and autogenous vein grafts. Differences were significant between elastic lamina and adventitial

scaffolds and between elastic lamina scaffolds and vein grafts at day 10, 20, and 30 ($p < 0.001$). Means and standard deviations are presented.

Figure 3. Monocyte adhesion to various matrix specimens and influence of sodium orthovanadate and PMA on monocyte adhesion. (**A**) En face fluorescent micrographs showing Hoechst 33258-labeled monocyte nuclei on adventitial and elastic lamina specimens. Scale: 100 μm. (**B**) Measurements of monocyte density on adventitial and elastic lamina specimens with and without PMA and sodium orthovanadate. Differences were significant between elastic lamina and adventitial specimens, between elastic lamina specimens with and without PMA, and between elastic lamina specimens with and without sodium orthovanadate at all observations times ($p < 0.001$). Means and standard deviations are presented.

Figure 4. Influence of PMA, ELDPs, and sodium orthovanadate on monocyte release of MCP-1. (**A**) Immunoblot showing PMA-stimulated monocyte release of MCP-1 and the suppression of PMA-stimulated MCP-1 release by a treatment with ELDPs. (**B**) Enhancement of monocyte MCP-1 release by a treatment with sodium orthovanadate in the presence of ELDPs (without PMA stimulation). IP: immunoprecipitation. IB: immunoblotting.

Figure 5. Binding of ELDPs to SIRP α. (**A**) Co-immunoprecipitation of ELDPs with SIRP α and phosphorylation of SIRP α. IP: immunoprecipitation. IB: immunoblotting. (**B**) Fluorescent micrographs showing the binding of FITC-conjugated ELDPs to monocytes. Control samples were incubated with an unrelated FITC-conjugated secondary antibody. Scale: 10 μm. (**C**) Binding competition of an anti-SIRP α antibody with FITC-conjugated ELDPs. Curve 1: control with a FITC-conjugated unrelated secondary antibody 10 μg/ml. Curve 2:

FITC-conjugated ELDPs 10 μg/ml. Curve 3 and 4: FITC-conjugated ELDPs 10 μg/ml with anti-SIRP α antibody 5 and 10 μg/ml, respectively.

Figure 6. Co-immunoprecipitation and phosphorylation of SIRP α and SHP-1, and the influence of anti-SIRP α antibody on the anti-inflammatory effects of ELDPs and elastic laminae. (**A**) Co-immunoprecipitation and phosphorylation of SIRP α and SHP-1. (**B**) Blockage of the inhibitory effects of ELDPs on monocyte MCP-1 release by a treatment with the anti-SIRP α antibody developed with the extracellular domain of SIRP α as an antigen. Ab.: anti-SIRP α antibody. IP: immunoprecipitation. IB: immunoblotting. (**C**) Influence of the anti-SIRP α antibody (Ab) on monocyte adhesion to elastic laminae. Differences between elastic lamina specimens with and without the anti-SIRP α antibody were significant at both times ($p < 0.001$). (**D**) Influence of the anti-SIRP α antibody (Ab) on monocyte adhesion to adventitia. Differences between adventitial specimens with and without the anti-SIRP α antibody were not significant at both times ($p > 0.05$). Means and standard deviations are presented in panels C and D.

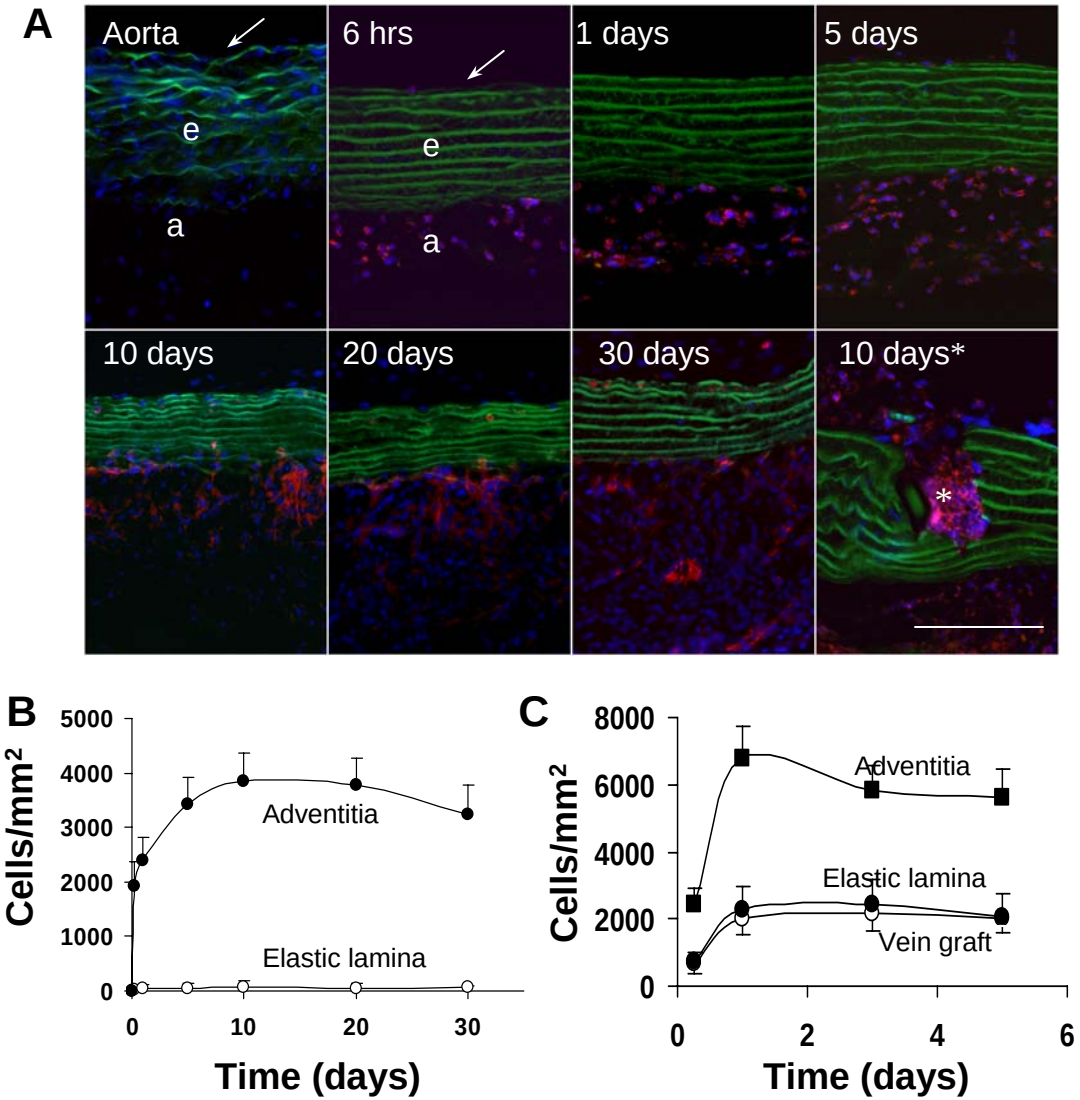
A
Aorta
e
a
6 hrs
e
a
1 days
5 days
10 days
20 days
30 days
10 days*
*
B
Cells/mm²
5000
4000
3000
2000
1000
0
Adventitia
Elastic lamina
0
10
20
30
Time (days)
C
Cells/mm²
8000
6000
4000
2000
0
Adventitia
Elastic lamina
Vein graft
0
2
4
6
Time (days)

Figure 1

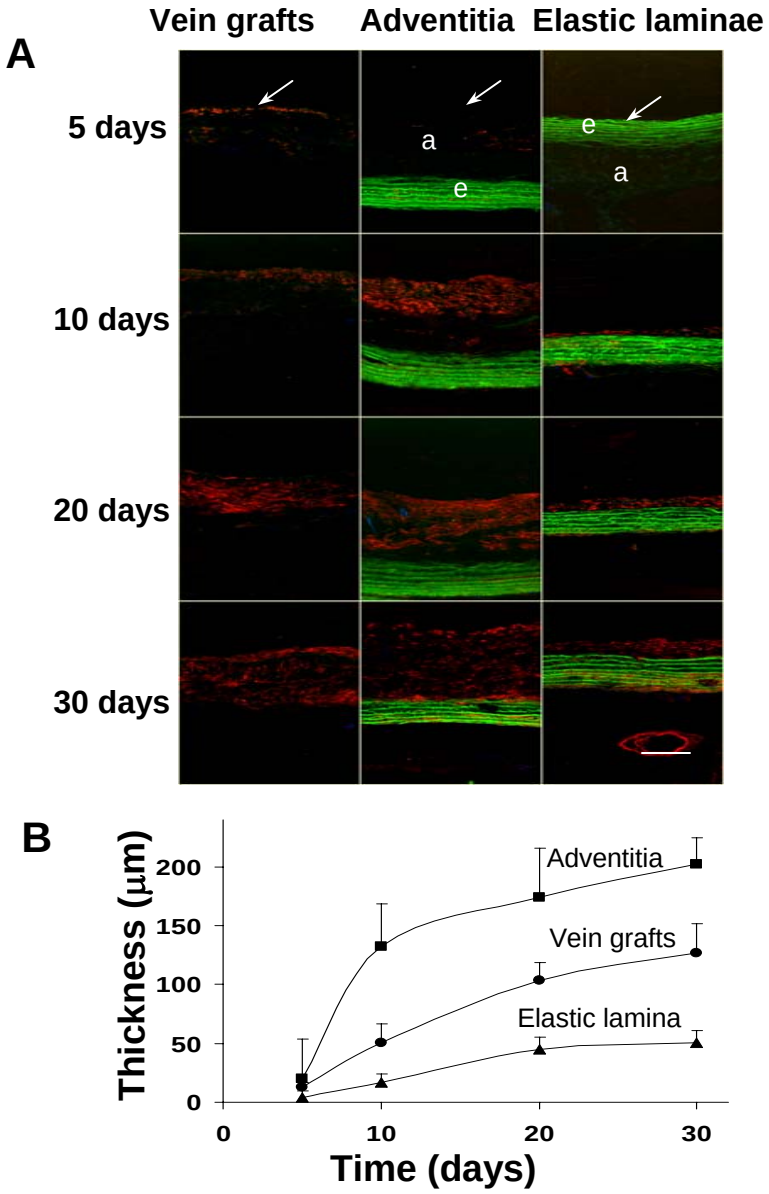
A
Vein grafts
Adventitia
Elastic laminae
5 days
10 days
20 days
30 days
a
e
e
a
B
Thickness (μm)
0
50
100
150
200
0
10
20
30
Time (days)
Adventitia
Vein grafts
Elastic lamina

Figure 2

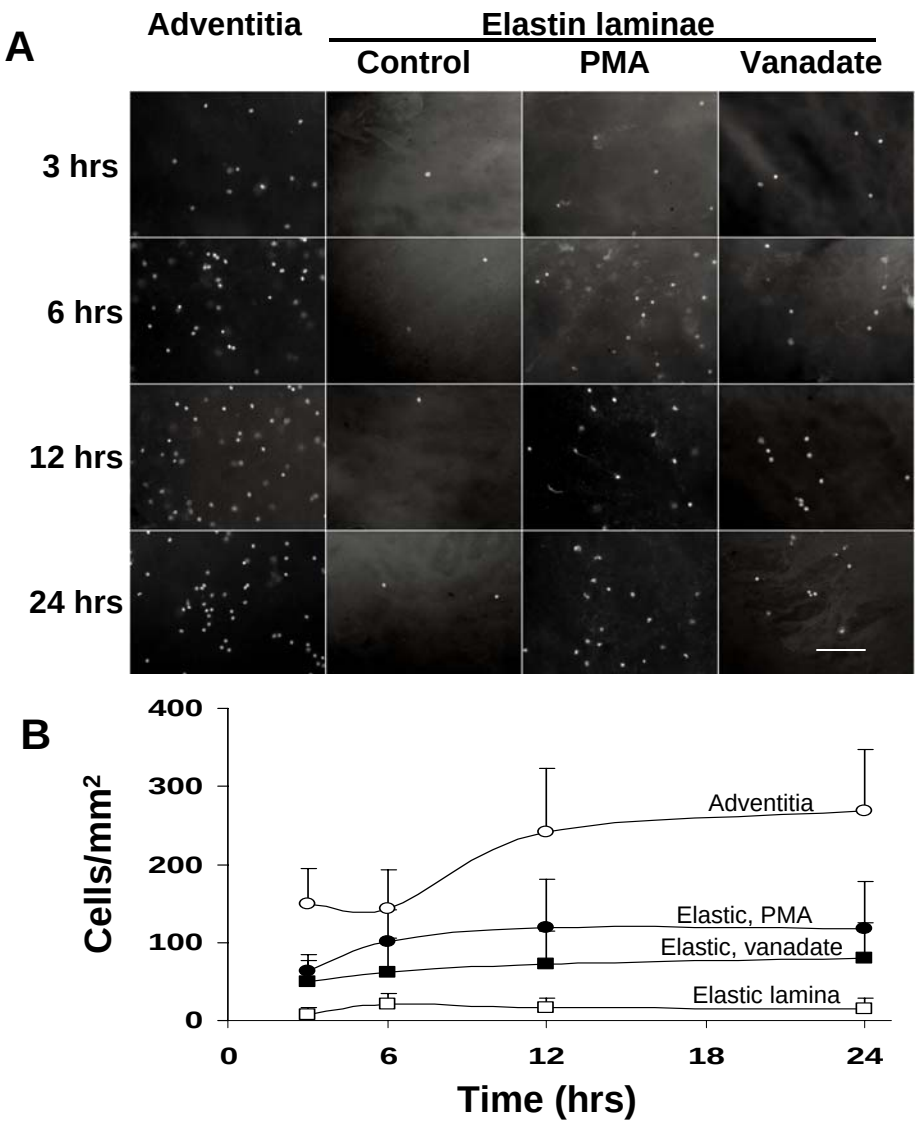
A
Adventitia
Elastin laminae
Control
PMA
Vanadate
3 hrs
6 hrs
12 hrs
24 hrs
B
Cells/mm²
400
300
200
100
0
Adventitia
Elastic, PMA
Elastic, vanadate
Elastic lamina
0
6
12
18
24
Time (hrs)

Figure 3

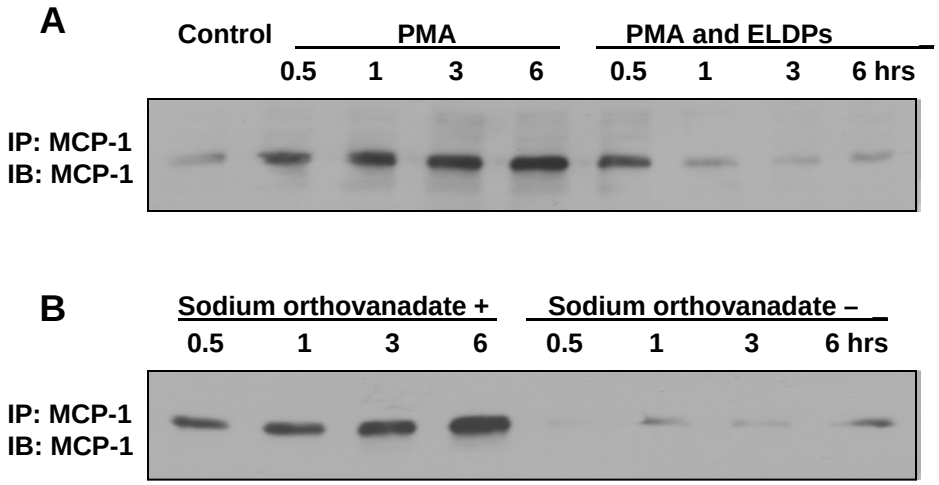
A
Control
PMA
PMA and ELDPs
0.5
1
3
6
0.5
1
3
6 hrs
IP: MCP-1
IB: MCP-1
B
Sodium orthovanadate +
Sodium orthovanadate –
0.5
1
3
6
0.5
1
3
6 hrs
IP: MCP-1
IB: MCP-1

Figure 4

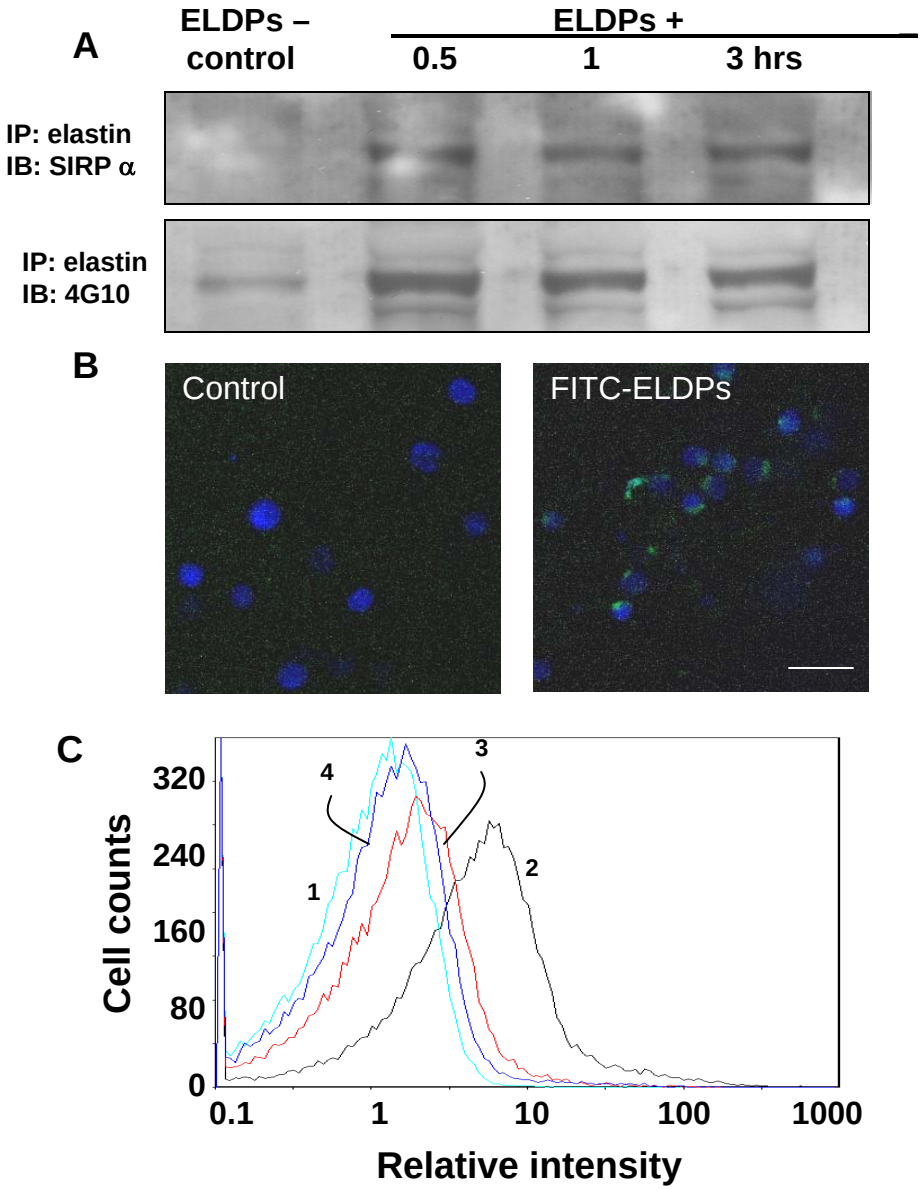
A
ELDPs –
control
ELDPs +
0.5
1
3 hrs
IP: elastin
IB: SIRP α
IP: elastin
IB: 4G10
B
Control
FITC-ELDPs
C
Cell counts
320
240
160
80
0
0.1
1
10
100
1000
Relative intensity
1
2
3
4

Figure 5

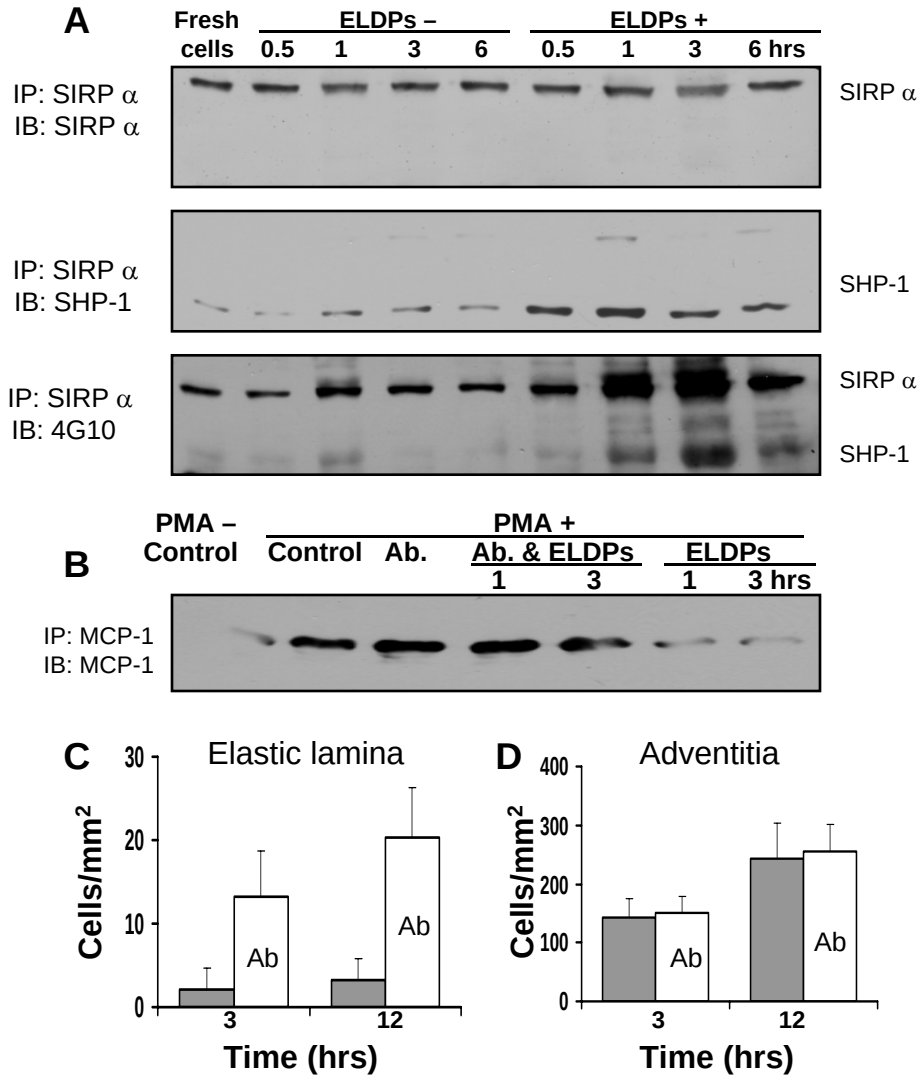
A
Fresh cells
ELDPs –
ELDPs +
0.5
1
3
6
0.5
1
3
6 hrs
IP: SIRP α
IB: SIRP α
SIRP α
IP: SIRP α
IB: SHP-1
SHP-1
IP: SIRP α
IB: 4G10
SIRP α
SHP-1
B
PMA –
Control
PMA +
Control
Ab.
Ab. & ELDPs
ELDPs
1
3
1
3 hrs
IP: MCP-1
IB: MCP-1
C
Elastic lamina
Cells/mm²
30
20
10
0
Ab
Ab
3
12
Time (hrs)
D
Adventitia
Cells/mm²
400
300
200
100
0
Ab
Ab
3
12
Time (hrs)

Figure 6