Interleukin-2 is present in human blood vessels
and released in biologically active form by heparanase

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Interleukin-2 (IL-2) is a multifaceted cytokine with immunostimulatory and immunosuppressive properties. Our laboratory recently demonstrated that the availability of IL-2 is regulated, in part, by association with perlecan, a heparan sulfate proteoglycan. Given the abundance of perlecan in blood vessels, we asked whether IL-2 is present in vessel walls. Our results indicate that IL-2 is associated with endothelial and smooth muscle cells within the human arterial wall. This IL-2 is released by heparanase, and promotes the proliferation of an IL-2-dependent cell line. Given the presence of IL-2 in human arteries, we asked whether the large vessels of IL-2-deficient mice were normal. The aortas of IL-2-deficient mice exhibited a loss of smooth muscle cells, suggesting that IL-2 may contribute to their survival. In their entirety, these results suggest a here-to-fore unrecognized role of IL-2 in vascular biology, and have significant implications for both the immune and cardiovascular systems. Immunology and Cell Biology (2012) 90, 159–167; doi:10.1038/icb.2011.45; published online 24 May 2011

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Interleukin-2 (IL-2) is a multifunctional cytokine, known to promote apoptosis, proliferation and survival of lymphocytes. The importance of IL-2 has recently resurfaced in the context of maintaining T-regulatory cells. Given the many functions of this cytokine, means by which the availability of IL-2 is regulated are of critical importance. Our laboratory previously reported that IL-2 is retained in lymphoid tissues by association with heparan sulfate (HS) glycosaminoglycan. In vivo, HS is usually found in proteoglycan form, in which the HS glycosaminoglycan chains are covalently linked to a specific core protein. We recently demonstrated that perlecan is the major proteoglycan that binds IL-2 in murine spleens. As perlecan is one of the main HS proteoglycans (HSPGs) found in blood vessels, we asked whether IL-2 is retained in human vessels.

Perlecan is a large HSPG, typically found pericellularly or in basement membranes. Both endothelial cells and smooth muscle cells, the major cells comprising vascular walls, produce perlecan. The HS chains of perlecan and other HSPGs have a significant role in several vascular wall functions, including the regulation of coagulation via binding to anti-thrombin III, recruitment of leukocytes via binding to chemokines and regulation of smooth muscle cell proliferation via binding to fibroblast growth factor-2.

Because of their location in subendothelial basement membranes, HSPGs such as perlecan, agrin and type XVIII collagen contribute to the barrier function of these structures. Leukocytes transmigrating between endothelial cells must degrade the basement membrane before entering the tissues. This degradation is achieved through enzymes such as various proteases and heparanase.

Heparanase is an endo-β-D-glucuronidase capable of cleaving heparin and the HS oligosaccharides of HSPGs. Many cells of the immune system have been reported to express heparanase, including T cells, B cells, neutrophils, macrophages, platelets, dendritic cells and endothelial cells. Heparanase not only facilitates the extravasation of leukocytes, but it potentially releases chemokines and/or growth factors sequestered in basement membranes by association with HS chains.

In light of the critical location of perlecan with respect to leukocyte-vascular interactions, the importance of IL-2 to immune function, and our previous report regarding the association of IL-2 with perlecan, we asked whether IL-2 is retained in the vascular wall of human blood vessels. We found that IL-2 is retained along the endothelium and in the media of the arterial wall. This IL-2 is released by the activity of endogenous heparanase and induces proliferation of an IL-2-dependent cell line. Our results suggest that IL-2 may be strategically located to modulate T lymphocytes and other leukocytes expressing the IL-2 receptor as they extravasate through blood vessel walls. In addition, the identification of IL-2 in human iliac vessels prompted us to examine the aortas of IL-2-deficient mice, which were found to lose smooth muscle cells over time and become aneurysmal. These findings suggest that IL-2 may be important in the survival of vascular smooth muscle cells and may affect the development of aneurysms in humans.

RESULTS

Localization of IL-2 within human arteries

To determine whether IL-2 is present in blood vessel walls, we examined sections of human iliac artery by immunofluorescence.

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To facilitate visualization of cells within the arterial wall, smooth muscle cells were labeled with an antibody-recognizing α-smooth muscle actin. Endothelial cells were localized via Ulex europaeus-1 (UEA-1), and the internal elastic lamina was visualized by autofluorescence. Co-localization studies were performed by confocal microscopy. As seen in Figure 1a, IL-2 was seen along endothelial cell surfaces and within the smooth muscle cell layer. The majority of IL-2 appeared to co-localize with smooth muscle cells (see insert, Figure 1) as very little actin without IL-2 was seen (green areas). Controls, including pre-incubation of the primary antibody with a five-molar excess of IL-2 (Figure 1b), and secondary antibody alone (not shown) were negative. A similar distribution of IL-2 was observed in murine aortas (not shown).

To ascertain whether the IL-2 seen in arteries was associated with HS, the sections were digested with commercially available heparinase I. Similar to heparanase, heparinase I degrades both heparin and HS oligosaccharides. As seen in Figure 1c, digestion of the tissue section with heparinase I before staining abrogated detection of IL-2 in the vessel wall. Widespread binding to the tissues of an anti-DHS antibody (HS stubs), specific for sugar moieties exposed after digestion of HSPGs with heparinase, confirmed the enzymatic cleavage of HS chains by heparinase I. Staining for cytokeratin 19 following digestion with heparinase I did not weaken demonstrably, suggesting that the loss of IL-2 was because of the activity of heparinase I, and not potential contaminating proteases. Finally, IL-2 was easily detectable if heparinase I was preincubated with the heparanase inhibitor PI-88 (Figure 1d), again indicating that the results observed were because of the enzymatic action of heparinase I. As PI-88 is a highly sulfated HS-like carbohydrate and competitive inhibitor of heparanase, the potential for PI-88 to elute IL-2 from

Figure 1 IL-2 is present in human blood vessel walls, and its detection is eliminated by heparinase digestion. (a) Sections of human iliac artery were labeled with antibodies recognizing IL-2 (tetramethylrhodamine isothiocyanate) and, in fluorescein isothiocyanate, either antibodies recognizing actin (smooth muscle cells), the lectin UEA (endothelial cells) or anti-perlecan antibodies, then assessed by confocal microscopy. Areas of co-localization are evident in yellow. Secondary controls were negative (not shown). Confocal images were generated using a Zeiss 510 Meta confocal microscope (Zeiss, Jena, Germany) using AIM software (Zeiss). (b) Sections of human iliac artery were stained with an anti-human IL-2 antibody (tetramethylrhodamine isothiocyanate) in the presence (pre-adsorbed IL-2) or absence (IL-2) of pre-incubation with a 5 M excess of human IL-2. (b–d) The green color originates from autofluorescence at 488 nm of elastin fibers within the internal and external elastic laminae. (c) Sections of human iliac artery were incubated for 2 h at 37 °C with heparinase I, or buffer alone, and then stained in tetramethylrhodamine isothiocyanate, with antibodies recognizing IL-2, HS stubs (sugar moieties exposed after digestion of HSPGs with heparinase) or cytokeratin 19 (CK-19). All stains were carried out in tetramethylrhodamine isothiocyanate. (d) Heparinase I was pre-incubated for 1 h with or without 150 ng PI-88, then incubated for 2 h at 37 °C as above. Control sections received PI-88 alone (PI-88) or buffer alone (see c, heparinase) under identical conditions. Sections were then stained for IL-2 (tetramethylrhodamine isothiocyanate). Tissue sections were mounted in Permount (Biomeda, Foster City, CA, USA) and visualized using a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan). Images were captured using a Cool Snap ES camera (Princeton Instruments, Trenton, NJ, USA) and processed using Metamorph image acquisition software (Molecular Devices Inc., Sunnyvale, CA, USA). Results shown are representative of 20 (a), 5 (b), 8 (c) and 3 (d) separate specimens. Scale bars, 25 μm (a) and 50 μm (b–d).
vessel walls was a concern. Tissue sections incubated with PI-88 alone showed robust staining for IL-2, suggesting that PI-88 was not eluting IL-2 from tissues.

In light of our recent work identifying perlecan as the major HSPG that binds IL-2 in murine spleen, we asked whether the distribution of IL-2 in human vessels co-localized with perlecan, as seen in Figure 1a. Perlecan was identified along smooth muscle and endothelial cell surfaces. IL-2 co-localized with perlecan in several areas, but there were also some areas in which IL-2 was present without perlecan (red versus yellow areas). These data suggest that IL-2 may bind other HSPGs, in addition to perlecan, present in vessel walls.

Identification of IL-2 in blood vessels by western blot analysis

As a second means of assessing the presence of IL-2 in blood vessels, we performed a western blot analysis on tissue homogenates of human arteries. Western blot analysis revealed a doublet at 17–19 kDa, consistent with IL-2 (Figure 2). Preadsorption of the anti-IL-2 antibody with a 5× excess of IL-2 eliminated recognition of the doublet.

We next asked whether heparinase digestion of arteries would release IL-2. To this end, small pieces of vessel were incubated with heparinase I, and the material liberated (digest) was assessed for IL-2 by western blot analysis. Digestion with heparinase I liberated a strong 17 kDa band, compared with the same band in the homogenate, which was relatively light (Figure 2a). These data suggest that the amount of IL-2 (relative to other proteins present) is fairly low in the homogenate but much higher in the heparinase digest.

In summary, these results suggest that IL-2 is retained in blood vessel walls by association with HS. Furthermore, this IL-2 is liberated by heparinase digestion. Release of IL-2 by heparinase is consistent with previous results from our laboratory, demonstrating that IL-2 associates with the glycosaminoglycan chains, rather than the protein core, of HSPG.

Source of IL-2

Given the substantial amount of IL-2 found in blood vessels, we began to address the source of IL-2 in these tissues. AS a portion of IL-2 produced during an immune response likely enters the systemic circulation, we first asked whether IL-2 in the bloodstream is retained in blood vessels. To address this question, we labeled commercial IL-2 with an infrared dye (infrared-IL-2). The infrared-IL-2 was then administered intraperitoneally to Balb/c mice. Aortas were harvested, proteins were extracted and separated by SDS-polyacrylamide gel electrophoresis, then analyzed on an infrared scanner. As seen in

Figure 2 IL-2 in vessels is acquired via the bloodstream and local production, retained by heparan sulfate and released by heparinase digestion. (a) Six 2×2 mm pieces of human iliac artery were incubated at 37°C with 2 U heparinase I for 18 h. Soluble and insoluble materials were separated by centrifugation, and soluble tissue was homogenized in 4× urea. The resulting homogenate (100 μg) and soluble material (digest; 50 μg) were then analyzed by western blot for the presence of IL-2. A higher amount of homogenate versus digestate was analyzed to facilitate visualization of the IL-2 band. The results shown are representative of 20 experiments. Secondary antibodies alone, and preabsorption of the primary antibody with a 5× excess of IL-2, were negative. (b) Balb/c mice were given 1 μg infrared-IL-2 daily by intraperitoneal injection for 8 doses, and killed at 3 days following the last dose. Tissues were processed as described for western blot analysis, and analyzed on an Odyssey infrared scanner. Results shown are representative of 10 experiments. (c) Balb/c mice were given a single dose of 1.5 μg infrared-IL-2, and killed at 2.5 days later. Five 1 mm long pieces of murine aorta were incubated at 37°C with 2 U heparinase I or heparinase buffer for 18 h, and the released material was separated by SDS-polyacrylamide gel electrophoresis and analyzed on an Odyssey infrared scanner. Results shown are representative of two experiments. IL-2 was labeled with infrared dye as described in Methods. (d) Murine aortas were harvested, thoroughly flushed and the adventitia was removed. Total RNA was extracted and assayed for the presence of IL-2 mRNA by reverse transcriptase PCR. A small piece of murine spleen was similarly processed for comparison. Expression of β actin was used as a loading control. The results shown are representative of three experiments. (e) Transgenic Balb/c mice expressing an IL-2 promoter/green fluorescent protein reporter were given 10 μg anti-CD3 by intraperitoneal injection. Tissues were harvested at 16 h later and processed for staining with anti-green fluorescent protein antibodies to enhance the green fluorescent protein signal. Results shown are representative of two separate experiments. Scale bar, 10 μm.
Figure 2b, infrared-IL-2 was identified in the aortic tissues. Although multiple doses of infrared-IL-2 were given in this particular experiment, a single dose of 1.5 μg of infrared-IL-2 also resulted in detectable infrared-IL-2 within vessels (Figure 2c). To determine whether this IL-2 was retained by HS, we digested the tissues with heparanase I. Digestion of the vessel tissue with heparanase I released the infrared-IL-2 (Figure 2c), suggesting that IL-2 gains access to vessels via the bloodstream, and is retained there by HS.

We next asked whether cells within the blood vessel wall produce IL-2. To this end, we first asked whether IL-2 message is expressed in blood vessel tissue. As seen in Figure 2d, IL-2 message was detected in murine aortas. This result suggests that at least some ‘intravascular’ IL-2 is produced within the vessel itself. As T cells are the main producers of IL-2, we asked whether T cells expressing IL-2 were present within aortic tissues. To address this question, we used transgenic mice that express green fluorescent protein on the activation of the IL-2 promoter. To promote IL-2 expression, the transgenic mice were first stimulated with anti-CD3. Aortas were harvested at 24 h later and analyzed by confocal microscopy. Murine aortas did contain scattered green fluorescent protein+ cells, indicating that T cells producing IL-2 are present in large vessels such as the aorta (Figure 2e).

In total, these results suggest that IL-2 in blood vessels comes both from systemic sources and local production, and that T cells provide at least one source of vascular IL-2. Whether cells comprising the vessel wall contribute to intravascular IL-2 is an interesting question that will require further study.

IL-2 in blood vessels promotes proliferation on release by endogenous heparanase

In light of the above results, we next asked whether the IL-2 released from human iliac arteries by heparanase is biologically active. To this end, we developed a bioassay using the IL-2-dependent T cell line CTLL-2, in which responses to IL-2 would be dependent on its release from arterial tissue. The first step in developing this assay was to determine whether CTLL-2 cells express the heparanase enzyme. Western blot analysis was performed on both cell lysates and media from CTLL-2 cultures. As seen in Figure 3a, both the pro (inactive, 65kDa) and active (51kDa) forms of the heparanase enzyme were present in the CTLL-2 cell lysates and culture supernatants.

To ensure that the heparanase expressed by CTLL-2 cells was active, a heparanase activity assay was performed using media from CTLL-2 cultures. As seen in Figure 3b, supernatants from CTLL-2 cultures exhibited heparanase activity that was inhibited by PI-88 (a heparanase inhibitor), as well as two distinct anti-heparanase antibodies.

In a tandem assay, an isotype control for the heparanase antibody was negative (CTLL-2-conditioned media 6132 ± 190 c.p.m., CTLL-2-conditioned media plus rabbit immunoglobulin G 5565 ± 468 c.p.m.). In a second tandem assay, the commercially available anti-heparanase antibody (anti-hepα1) was demonstrated to have blocking activity, as heparanase from a heparanase-producing cell line elicited 2600 ± 830 c.p.m., and heparanase plus anti-heparanase antibodies generated only 91 ± 21 c.p.m.

Having confirmed that CTLL-2 cells produce active heparanase, we proceeded to ask whether this heparanase would release sufficient quantities of biologically active IL-2 to induce proliferation. To this end, CTLL-2 cells were cultured with a small piece of human iliac artery placed in a transwell insert. The transwell membrane ensured that the cells were physically separated from the tissues and could, therefore, respond only to soluble IL-2 liberated by the actions of heparanase and/or other enzymes released by the CTLL-2 cells. As seen in Figure 3c, CTLL-2 cells proliferated when co-cultured with, but separated from, pieces of blood vessel. Inhibition of heparanase by an anti-heparanase antibodies or PI-88 abrogated the proliferative response. A blocking anti-IL-2 antibody also inhibited proliferation, indicating that the proliferation observed was because of IL-2. Isotype controls for both the anti-heparanase and anti-IL-2 antibodies had no effect on proliferation (not shown). In addition, PI-88 did not influence the proliferation of CTLL-2 cells cultured with IL-2 (3.2 ng ml⁻¹ IL-2 146 285 ± 1088 c.p.m., 3.2 ng ml⁻¹ IL-2 +3 ng ml⁻¹ PI-88 151 320 ± 7497 c.p.m.).

Given the widespread presence of IL-2 along endothelium and in the parenchyma of lymphoid tissues in vivo (Figure 1), lymphoid cells may be in frequent contact with IL-2. Therefore, we asked whether proliferation of CTLL-2 cells in direct contact with blood vessel tissue (that is, in the absence of the transwell) was dependent on release of IL-2 by heparanase. As seen in Figure 3d, proliferation of CTLL-2 cells in direct contact with vessel tissue was inhibited by anti-heparanase antibodies, suggesting that IL-2 must be released by heparanase to promote proliferation. Although our findings indicate that heparanase releases IL-2, they do not preclude the possibility that other enzymes, such as matrix metalloproteinases, release IL-2 as well. The extent to which inhibition of heparanase decreased proliferation of the CTLL-2 cells, however, suggests that heparanase is the main enzyme responsible for releasing IL-2 from HSPGs.

IL-2-knockout (KO) mice exhibit aneurysmal aortas

Although IL-2 is typically thought of as cytokine that affects lymphocytes, in light of the significant amount of IL-2 found in human iliac arteries, we began to determine whether IL-2 might influence the cells comprising the vascular wall. To this end, aortas of IL-2 KO mice were examined histologically and compared with age-matched, wild-type (WT) controls. The IL-2 KO mice used were DO11.10/IL-2 KO and DO11.10/RAG-1 KO/IL-2 KO mice. The latter mice express only T cells bearing the transgenic T-cell receptor, DO11.10, and therefore lack autoimmunity. Using these mice alleviates the potential influence of autoimmune-mediated inflammatory changes on vessels.

At relatively young ages (6–10 weeks), the aortas of IL-2 KO mice were similar to WT mice (not shown). By ~20 weeks of age and older, the aortas of the IL-2 KO mice exhibited a loss of smooth muscle cells (Figure 4a). In addition, the morphology of the smooth muscle cells was different. In the IL-2 KO mice, the cell bodies of the smooth muscle cells were smaller and more compact, whereas in WT mice the smooth muscle cells were larger with more extended processes (see ×40 and inserts, Figure 4). In severe cases (Figure 4a, KO), the aortas became extremely thin with very few smooth muscle cells remaining. Morphometric analysis of Van Gieson-stained WT and IL-2 KO aortas revealed that the internal elastic laminae within the IL-2 KO vessels were closer together than the WT, likely because of the smaller smooth muscle cell bodies in the KO mice (Figure 4b). Elastin fibers in the adventitia were abnormal as well. In the WT mice, these fibers appeared as thin, pale strands. In the KO mice, these fibers had a thick, beaded appearance and were much darker in color.

The endothelial cell layer also appeared abnormal (Figure 4a). Although in the WT mice the endothelial layer was smooth and without breaks, the IL-2-deficient mice exhibited irregular endothelial cell layers with gaps in which no endothelial cells were present. Aortas from IL-2 receptor β (IL-2Rβ) KO mice also exhibited similar changes (not shown). Although this finding is likely an artifact of tissue preservation, it does suggest that endothelial cells or the endothelial cell–basement membrane associations of IL-2-deficient mice are...
fragile, as compared with WT mice whose endothelial layer remained intact following vessel harvest and histological processing. These findings suggest that IL-2, either directly or indirectly, influences vascular smooth muscle cells. Given the abnormalities seen throughout the vessel wall, IL-2 may affect other cell types as well. Whether this represents an impact on factors such as survival, proliferation, adhesivity, apoptosis or response to mechanical stress, remains to be determined.

Murine vascular smooth muscle cells express IL-2Rβ

In order for IL-2 to exhibit a direct effect on cells comprising the vascular wall, these cells must express the IL-2R. Although reports in the literature indicate that both endothelial cells and fibroblasts express IL-2Rα and β, there are no convincing studies regarding the expression of IL-2R on vascular smooth muscle cells. Therefore, we cultured smooth muscle cells from murine aortas, and tested the cell lysates for expression of IL-2Rβ by western blot analysis. As seen in Figure 5a, murine vascular smooth cells expressed IL-2Rβ. These results were not because of the contamination of the cultures with endothelial cells or fibroblasts, as expression of UEA-1 (endothelial cell marker) and S100A4 (fibroblast marker) were negative. Human smooth muscle cells in aortic tissue (Figure 5c) and cultures (Figure 5d) were also found to express IL-2Rβ protein and message, respectively. These results suggest that IL-2 has a direct effect on smooth muscle cells via the IL-2R. Whether this effect is via the tripartite receptor or IL-2 receptor is a question for future studies.

DISCUSSION

Lymphocytes must pass through blood vessels to enter inflamed tissues. To cross the subendothelial basement membrane, extravasating lymphocytes release proteases and heparanase. Our finding that IL-2 is present on the surface of endothelial cells and smooth muscle cells lining blood vessels, and that biologically active IL-2 is released by heparanase, suggests that IL-2 has an unanticipated early role in modulating immune responses in inflamed tissues. Although for logistic reasons our studies used large vessels, IL-2 is retained along the endothelium lining entry points into the spleen, including both the marginal zone and central arteriole.
In their review on endothelial injury, Tesfamariam and DeFelice state,25 ‘The endothelium comprises the largest homogeneous surface of the body for actively mediating immune defense’. The localization of IL-2 along vascular endothelium, therefore, suggests that IL-2 may be a critical ‘first responder’ in immune defense mechanisms. IL-2 is typically not thought of in these terms as, depending on the antigen and route, significant IL-2 production by T cells is not observed until anywhere from 15 to 45 h after antigen exposure.26,27

Although the functions of IL-2 are usually associated with lymphocytes and natural killer cells, IL-2 is known to influence other cells important to normal immune responses, including neutrophils,28 dendritic cells,29 macrophages30 and platelets.31 Interestingly, IL-2 has been shown to increase the adhesion of neutrophils, natural killer cells and lymphocytes to endothelial cells.28,32,33 Administration of IL-2 in vivo has been shown to induce microvascular platelet thrombi via increased endothelial–platelet interactions.31 Therefore, IL-2 localized to blood vessels may influence cells responsible for both innate and acquired immune responses as they enter inflamed tissues. In addition, local release of IL-2 in vessels may potentially contribute to the development of microthrombi in pathologies, such as hemolytic uremic syndrome and antibody-mediated rejection, which are characterized by endothelial cell damage.25,34

Our studies suggest that the IL-2 retained in blood vessels must first be released by heparanase to stimulate proliferation, as anti-heparanase antibodies inhibited the proliferation of CTL.2 cells cultured directly with vessel tissue (see Figure 3d). As mentioned in the introduction, heparanase is produced by most immune cells, including lymphocytes, platelets, neutrophils, endothelial cells and

Figure 4 The aortas of IL-2-deficient mice exhibit several abnormalities. (a) Sections of murine aortas were double labeled with smooth muscle cell actin (fluorescein isothiocyanate) and, to identify endothelial cells, anti-CD31 (tetramethylrhodamine isothiocyanate). Aortas from 24-week-old D011.10/IL-2+/+(WT) mice were compared with aortas from 27-week-old D011.10/IL-2−/− (IL-2 KO) and D011.10/RAG−/−/IL-2−/− (DKO) mice. The vascular lumen is identified with an L. A digital zoom image of the smooth muscle cell identified by an arrow is shown as an insert. Because the extremely abnormal IL-2 KO aortas were ectatic and fragile, the lumens often collapsed on themselves when harvested (see DKO) and the endothelial layer was prone to lifting away from the smooth muscle cells. The results shown are representative of approximately five IL-2 KO and two IL-2 DKO mice of ages similar to those above. Tissue sections were imaged as described in Figure 1b. Scale bars, 50 μm (×20), 25 μm (×40). (b) Pieces of murine aortas from WT and IL-2 KO mice were preserved in 4% formalin and embedded in paraffin blocks. Sections (5 μm) were cut, deparaffinated and then stained with Verhoeff van Gieson stain. Two different WT and IL-2 KO aortas of comparable ages are shown (WT 36 and 48 weeks old; IL-2 KO 34 and 35 weeks old). Tissue sections were imaged as described in Figure 1b. The percentage of interlaminar space (interlaminar area/laminae+interlaminar area)100) was significantly greater in IL-2 WT mice (P<0.0001). Scale bar, 25 μm.
Heparanase is stored in active form in lysosomes, then released by degranulation in response to chemoattractants or inflammatory stimuli.\(^\text{20}\)

Despite its production by multiple cell types, heparanase is tightly controlled by several mechanisms, including (1) regulated secretion, (2) uptake of secreted heparanase by low-density lipoprotein-related receptor protein\(^\text{14}\) and syndecan-4\(^\text{35}\) and (3) expression, in that only one functional isoform of mammalian heparanase has been identified to date.\(^\text{14}\) Therefore, even though IL-2 is apparently widely distributed along major vessels and smaller intraparenchymal vessels,\(^\text{3,4,22}\) its release by heparanase would be tightly controlled by the above mechanisms. Conversely, dysregulation of heparanase contributes to the metastatic capabilities of many cancers. For this reason, heparanase is being investigated as target of chemotherapeutic agents in both animal models and human clinical trials.\(^\text{36}\) Whether release of IL-2 by heparanase contributes to the metastatic potential of certain cancers, especially leukemias and lymphomas, remains to be determined.

HS binds several types of biologically active proteins, including specific cytokines, chemokines and growth factors.\(^\text{15}\) Although it is logical to postulate that these various mediators are released by heparanase, only a few examples in the literature validate this assumption. The primary example of a heparanase-mediated release of a growth factor is that of basic fibroblast growth factor.\(^\text{37}\) Basic fibroblast growth factor is sequestered and protected in the subendothelial matrix by HSPGs. Heparanase, released by activated platelets (and likely other cells) at the site of tissue injury, releases basic fibroblast growth factor, which in turn stimulates smooth muscle cell migration and proliferation.\(^\text{37}\) Despite a paucity of information regarding release of growth factors by heparanase, several examples of protease-mediated release of growth factors exist. Some of these include the release of transforming growth factor \(\beta\) by chymases and elastases,\(^\text{38}\) the release of colony-stimulating factor-1 by proteases\(^\text{39}\) and the release of insulin-like growth factor II by matrix metalloproteinase 7.\(^\text{40}\) Although our data demonstrate that HSPG-bound IL-2 is released by heparanase, other enzymes, including various proteases, may release IL-2 via cleaving the protein core of the proteoglycan.

As T cells are the primary producers of IL-2 in the immune system, these cells are likely the main source of the IL-2 observed in human arteries. Although the presence of T cells in atherosclerotic vessels is well documented, Galkina et al.\(^\text{41}\) showed that a substantial number of T cells, B cells, dendritic cells and macrophages are present in normal vessels. Our studies indicate that IL-2-producing T cells are present within arterial walls (Figure 2d). It is possible, however, that some IL-2 comes from the cells comprising the blood vessel wall, as both endothelial cells\(^\text{42}\) and smooth muscle cells\(^\text{43}\) have been reported to express IL-2 under certain conditions. Our studies with infrared-IL-2 suggest that circulating IL-2 is readily taken up in vessels, indicating that some portion of IL-2 in vessels comes from IL-2 that enters the micro- or macrocirculation. This uptake of IL-2, evident in vessels and other tissues (Figure 2 and unpublished data), likely contributes to the short half-life of IL-2 in the bloodstream.\(^\text{44}\)

The presence of IL-2 in both large and small vessels raises the question as to whether IL-2 has a direct influence on endothelial cells and smooth muscle cells. Previous studies have shown that IL-2 has a direct effect on the permeability of endothelial cells.\(^\text{53}\) A recent study by Bae et al.\(^\text{53}\) indicated that IL-2 promotes angiogenesis, and demonstrated that the endothelial cells express IL-2R\(\alpha\) and \(\beta\). IL-2 has also been shown to stimulate glycosaminoglycan synthesis in vascular smooth muscle cells and enhance their responsiveness to...
angiotensin II. Our histological examination of aortas from IL-2-deficient mice suggests that IL-2 has a role in maintaining vascular smooth muscle cells. As the loss of smooth muscle cells was also seen in DO11.10/RAG-1 KO/IL-2 mice, which do not exhibit autoimmunity, this pathology is not the result of nonspecific, autoimmune-mediated inflammation. Our finding that vascular smooth muscle cells express IL-2Rβ suggests that IL-2 has a direct affect on these cells; however, the mechanism(s) by which IL-2 mediates this affect remains to be determined. Clearly, the influence of IL-2 on smooth muscle cells suggests exciting possibilities and warrants further study.

In summary, the identification of HS-bound IL-2 in human blood vessels is unprecedented and has several implications for the role of IL-2 in initiating immune responses. In addition, this finding leads to the consideration of alternative functions for IL-2 outside the immune system. It is both daunting and refreshing to discover that new biology is yet to be learned regarding one of oldest cytokines of immunology.

**METHODS**

**Materials and tissues**

Small sections of human iliac artery and spleen were obtained from deceased donor organs. Consent for research was provided by next of kin. Pieces of murine aorta were obtained from DO11.10, DO11.10/IL-2 KO and DO11.10/RAG-1 KO/IL-2 KO mice present within our colony. Balb/c DO11.10 mice expressing a transgenic IL-2 promoter/green fluorescent protein reporter were the kind gift of Dr Casey Weaver.13 Mice were housed in specific-pathogen-free facilities and all experiments were in accordance with the protocols approved by the University of Nebraska Institutional Animal Care and Use Committee. Additional methodology

**Vascular smooth muscle cell cultures**

Murine aortas were harvested and placed in glass petri dishes containing dissection media (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered saline). After removal of extraneous tissue, the aortas were incubated for 30 min at 37°C in an enzyme solution composed of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered saline containing 2 mg ml⁻¹ bovine serum albumin, 1 mg ml⁻¹ collagenase type I, 0.375 mg ml⁻¹ soybean trypsin inhibitor and 0.125 mg ml⁻¹ elastase type III (Worthington Biochemical Corporation, Lakewood, NJ, USA). The adventitia was then removed, and the denuded aortas were minced into small pieces, and placed in fresh enzyme solution for 1.5–2 h with slight agitation. After dissociation, cells were pelleted by centrifugation, washed three times with Dulbecco’s modified Eagle’s medium (high glucose media containing 10% fetal bovine serum and plated in T-25 flasks that are maintained at 37°C and 5% CO₂. Cultured cells were used after three passages. For western blot analysis, cells were scraped into ice-cold 8 M urea and lysates were analyzed as described below.

**Transwell assay**

CTLL-2 cells, 1×10⁶ ml⁻¹, were cultured in RPMI 1640 media with 1% fetal bovine serum. Transwell inserts (Nunc, Rochester, NY, USA), each containing one 2×2 mm (8 μg) piece of iliac artery, were used to provide a physical separation between the cells and the tissue. The CTLL-2 cells were first plated in the wells and the tissue was placed in the transwell insert. Anti-heparanase antibodies, PI-88 or neutralizing anti-IL-2 antibodies were added to the culture media as indicated in the figure legend (Figure 4c). Proliferative responses were assessed as previously described.3 Briefly, the CTLL-2 cells were cultured for 96 h at 37°C and [³H] thymidine, 1 μCi ml⁻¹, was added during the last 8 h of the culture period. Wells were then harvested using a cell harvester (Skatron Instruments, Lier, Norway), and the samples counted in a scintillation counter.

**Heparanase activity assay**

Heparanase activity was tested in culture supernatants from CTLL-2 cells. PI-88 or anti-heparanase antibodies were pre-incubated with select samples for 1 h at 24°C as indicated in the figure legend. [³H] heparin conjugated to agarose beads (10,000 c.p.m. per reaction; generously provided by Dr Jeffery Platt) was resuspended in the sample to be tested, incubated at 37°C for 2 h and then centrifuged. [³H] heparin cleaved by heparanase was then detected in the supernatant by scintillation counting. CAG myeloma cells, transfected with heparanase, were used as a source of heparanase as a positive control.47

**Dye-labeled IL-2**

Murine IL-2 (R & D Systems) was labeled with an activated infrared dye (800CW, LI-COR Biosciences, Lincoln, NE, USA) per the manufacturer’s instructions. Briefly, activated infrared dye was added to the IL-2 at a molar ratio of 1:1. Following a 2 h incubation at 24°C, unconjugated dye was removed from the preparation using a mini de-salting column. The concentration of the dye-conjugated IL-2 was then determined by Bradford assay (Sigma-Aldrich). To confirm that the IL-2 was covalently conjugated to the 800CW dye, 0.5 μg of dye-IL-2 was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and analyzed on an Odyssey infrared scanner (LI-COR Biosciences).

**Morphometry on aortic tissue sections**

The space between aortic wall laminae was measured using Adobe Photoshop 7 software. The area of lesions was measured using ImageJ software. For western blot analysis, cells were scraped into ice-cold 8 M urea and lysates were analyzed as described below.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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**Author contributions:** JDM designed and performed the experiments and analyzed the data. SEC and DRS performed the experiments and analyzed data. RBS performed the morphometric analysis. LEW designed the experiments, analyzed the data and wrote the manuscript.
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