Anti-clotting functions of lymphatics form the natural on-off switch for immune recognition by controlling the antigens and immune cells access to the lymph nodes

The ability of lymph to clot indicates that similar to blood vessels lymphatics must have means to counteract this process. We analyzed their hemostatic properties, tailoring them for the potential therapeutic applications. Inflammatory stimuli induced tissue factor-dependent focal lymph clotting while blocking thrombomodulin lead to widespread but also transient occlusion of collecting vessels. Decellularization of lymphatics resulted in tissue factor-independent lymphatic occlusion by widespread and persistent lymph clots. These decellularized basement membrane remnants of collectors were capable of macromolecules drainage and leukocytes transit only when lymph clotting was inhibited with heparin. In occluded ‘ghost’ vessels, fibrin was replaced with transient basement membrane-rich inclusions to be eventually reperfused. During regeneration, ghost vessels were filled with granuloma-like clusters of antigen-presenting and T cells. Despite that, immune response against allografts placed under non-drained skin was not developed as long lymphatics remained occluded, and graft survival was prolonged together with the delay of lymphatic regeneration with anti-angiogenic therapy. Other potential applications of lymphatic hemostatic control functions include blocking pathogen or metastasis spread but also should be considered in the treatment of lymphedema.

Intima lining of blood vasculature forms the only known surface that is able to control blood liquidity dynamically, and despite ontogenetic and functional similarities, that function has not been yet ascribed to its relative circulation of lymphatic vessels. However, since the first description of the lymphatic system by Olaf Rudbeck over 350 years ago, lymph is known for its ability to coaggregate when stored outside of the lymphatic vessels (discussed in 2,3,4). Interestingly, the above references illustrate that most of the research on lymph clotting was conducted over 50 years ago with little impact on the understanding of lymphatic related pathologies or therapeutic application. Indeed, even though lymph clotting time is delayed as compared to blood, it has intrinsic clotting properties. Its clotting time varies in a broad, up to 20-times range as a concentration of lymph clotting factors that fluctuate between species, individuals or even sampling location, and is 10 to 70% lower as compared to the blood. Together with the absence of platelet-derived anionic phospholipids required for activation of Xase and prothrombinase and a higher concentration of fibrinolytic factors assure lymph hypercoagulability under physiological conditions. Also, due to the lack of thrombocytes, lymph, in contrast to blood clots, do not retract. On the other hand, relatively slow lymph flow and possibility to substitute platelet phospholipids with membrane components of endothelial cells may favor lymph clotting for example, during inflammation, when augmented blood vessel permeability to plasma proteins leads to 3-fold enrichment of plasma proteins in interstitial fluid and lymph. While data are missing for the inflammation or injury-driven concentration change of individual clotting factors in the lymph, it is rather safe to assume that concentration of coagulation components would rise when the rate of protein extravasation from blood vessels increases up to 100 times normal versus 2,15,16.

These observations confirm that lymph is a hemostatic fluid, but more importantly, it infers that similar to blood vessels, lymphatics must have specific anti-coagulatory properties that enable them to control lymph fluidity. By doing so, the lymphatic system could regulate other passive functions of lymphatics, i.e., fluid drainage and cell trafficking, and similar to blood vessels use pro-hemostatic mechanisms to limit the spread of infections. However, only a handful of studies address thrombo-regulatory properties of lymphatic endothelium (for review see57). For example, it was shown that similar to blood endothelial cells; lymphatic collectors express thrombomodulin, a membrane receptor capable of switching the substrate specificity of thrombin to protein C, thus making thrombin a potent anticoagulant factor. On the other hand, lymphatic endothelium does not produce von Willebrand factor, a multimeric protein that participates in platelet activation on the exposed subendothelial collagen. This phenotype, however, is in line with the lack of platelets in lymph.

In response to pro-inflammatory molecules such as cytokines TNFa, IL-1β or TLR agonist LPS, blood endothelium can turn into a pro-thrombotic surface, e.g., by initiating the expression of tissue factor (TF), membrane receptor initiating extrinsic coagulation pathway. Few in vitro studies have demonstrated that lymphatic endothelial cells are also capable of responding to inflammatory stimuli, for example with increased synthesis of plasminogen activators and inhibitors12, 21.
In contrast to blood circulation, where vessel occlusion by thrombus leads to tissue ischemia and immediate localised symptomatic pain, development of edema that follows lymphatic blockage can take a long time and still remain undetected. Yet, there is clinical evidence of pathological lymph clotting that accompanies certain inflammatory pathologies. For example, intralymphatic lymph clots encapsulating the granuloma were found in the chronic inflammation,

surrounding dead worms in lymphatic filariasis or secondary lymphedema. These clots often undergo fibrosis, forming long-lasting intravascular intrusions in chronic diseases associated with human tumors or identified by us lymph fibrosis in an experimental mouse model of B16-F10 melanoma (Supplementary Fig. 1).

The transport of clottable lymph requires anchoring and co-localized with high expression of TF on lymphatic endothelium. These results indicated that thrombin does not induce lymph clotting directly but instead it activates certain cell clusters in divergent endothelium of collectors.

Heterogeneity of TF expression resembles the similar discontinuous pattern of CCL21 expression previously reported by our group but is also characteristic for blood endothelial cells where TF density on the cell surface varied strongly among TNFα-stimulated endothelial cells in vitro and in vivo.

Combination treatment with cytokines and thrombin had a cumulative effect on dynamic state the endothelium of lymphatic collector, similar to blood endothelial cells, express anti-thrombotic thrombomodulin receptor but do not express tissue factor (Fig. 2B). However, contrary to blood endothelium, lymphatic collector endothelium is not expected to encounter platelets, and consequently, they lack the intracellular stores of von Willebrand factor, the critical factor for platelet adhesion to the subendothelial collagen at the location of lymphatic wounds. Thus, by de novo expression of tissue factor on lymphatic endothelial cells appeared as the most intuitive and feasible approach. We assayed the activation of intralympatic coagulation in mouse skin by posttreatment intravenous injection of fluorescently labeled fibrinogen (Fig. 1F). Fibrinogen that leaked to the tissue was polymerized into insoluble fibrin, its fluorescent deposits could be detected with intravital microscopy of thin mouse ear or, after fixation, in the whole mount preparation with confocal optical sectioning.

TNFα and IL-1β are cytokines known as potent inducers of tissue factor (TF) expression in vitro but only on subsets of blood endothelial cells in vivo. Inflammation stimulation of TF on the inner layer of blood vessels results from the innate immune system and persistent, local vessel occlusion by thrombus. Similar in lymphatics, even in the absence of platelets, repeated intradermal injections of TNFα induced dot formation within draining collectors but only at discrete locations (Fig. 1C). No cumulative effect was observed when TNFα was co-injected with IL-1β, and this treatment produced a similar sparse distribution of clots within collectors. Activation of G-protein coupled receptor (GPR) 18 on inflammatory cells only removes the endothelial source of anti-thrombotic factors (thrombomodulin, plasminogen activators) but also expose negatively charged basement membranes, which laminin and collagen I can activate intrinsic coagulation cascade.

Induced TF expression on lymphatics leads to lymph clotting and obstruction of drainage. Here we report that clottable properties of lymphatics and the means to control them. In a normal physiological state the endothelium of lymphatic collector, similar to blood endothelial cells, express anti-thrombotic thrombomodulin receptor but do not express tissue factor (Fig. 2B). However, contrary to blood endothelium, lymphatic collector endothelium is not expected to encounter platelets, and consequently, they lack the intracellular stores of von Willebrand factor, the critical factor for platelet adhesion to the subendothelial collagen at the location of lymphatic wounds. Thus, by de novo expression of tissue factor on lymphatic endothelial cells appeared as the most intuitive and feasible approach. We assayed the activation of intralympatic coagulation in mouse skin by posttreatment intravenous injection of fluorescently labeled fibrinogen (Fig. 1F). Fibrinogen that leaked to the tissue was polymerized into insoluble fibrin, its fluorescent deposits could be detected with intravital microscopy of thin mouse ear or, after fixation, in the whole mount preparation with confocal optical sectioning.

TNFα and IL-1β are cytokines known as potent inducers of tissue factor (TF) expression in vitro but only on subsets of blood endothelial cells in vivo. Inflammation stimulation of TF on the inner layer of blood vessels results from the innate immune system and persistent, local vessel occlusion by thrombus. Similar in lymphatics, even in the absence of platelets, repeated intradermal injections of TNFα induced dot formation within draining collectors but only at discrete locations (Fig. 1C). No cumulative effect was observed when TNFα was co-injected with IL-1β, and this treatment produced a similar sparse distribution of clots within collectors. Activation of G-protein coupled receptor (GPR) 18 on inflammatory cells only removes the endothelial source of anti-thrombotic factors (thrombomodulin, plasminogen activators) but also expose negatively charged basement membranes, which laminin and collagen I can activate intrinsic coagulation cascade.

Induced TF expression on lymphatics leads to lymph clotting and obstruction of drainage. Here we report that clottable properties of lymphatics and the means to control them. In a normal physiological state the endothelium of lymphatic collector, similar to blood endothelial cells, express anti-thrombotic thrombomodulin receptor but do not express tissue factor (Fig. 2B). However, contrary to blood endothelium, lymphatic collector endothelium is not expected to encounter platelets, and consequently, they lack the intracellular stores of von Willebrand factor, the critical factor for platelet adhesion to the subendothelial collagen at the location of lymphatic wounds. Thus, by de novo expression of tissue factor on lymphatic endothelial cells appeared as the most intuitive and feasible approach. We assayed the activation of intralympatic coagulation in mouse skin by posttreatment intravenous injection of fluorescently labeled fibrinogen (Fig. 1F). Fibrinogen that leaked to the tissue was polymerized into insoluble fibrin, its fluorescent deposits could be detected with intravital microscopy of thin mouse ear or, after fixation, in the whole mount preparation with confocal optical sectioning.

TNFα and IL-1β are cytokines known as potent inducers of tissue factor (TF) expression in vitro but only on subsets of blood endothelial cells in vivo. Inflammation stimulation of TF on the inner layer of blood vessels results from the innate immune system and persistent, local vessel occlusion by thrombus. Similar in lymphatics, even in the absence of platelets, repeated intradermal injections of TNFα induced dot formation within draining collectors but only at discrete locations (Fig. 1C). No cumulative effect was observed when TNFα was co-injected with IL-1β, and this treatment produced a similar sparse distribution of clots within collectors. Activation of G-protein coupled receptor (GPR) 18 on inflammatory cells only removes the endothelial source of anti-thrombotic factors (thrombomodulin, plasminogen activators) but also expose negatively charged basement membranes, which laminin and collagen I can activate intrinsic coagulation cascade.

Induced TF expression on lymphatics leads to lymph clotting and obstruction of drainage. Here we report that clottable properties of lymphatics and the means to control them. In a normal physiological state the endothelium of lymphatic collector, similar to blood endothelial cells, express anti-thrombotic thrombomodulin receptor but do not express tissue factor (Fig. 2B). However, contrary to blood endothelium, lymphatic collector endothelium is not expected to encounter platelets, and consequently, they lack the intracellular stores of von Willebrand factor, the critical factor for platelet adhesion to the subendothelial collagen at the location of lymphatic wounds. Thus, by de novo expression of tissue factor on lymphatic endothelial cells appeared as the most intuitive and feasible approach. We assayed the activation of intralympatic coagulation in mouse skin by posttreatment intravenous injection of fluorescently labeled fibrinogen (Fig. 1F). Fibrinogen that leaked to the tissue was polymerized into insoluble fibrin, its fluorescent deposits could be detected with intravital microscopy of thin mouse ear or, after fixation, in the whole mount preparation with confocal optical sectioning.

TNFα and IL-1β are cytokines known as potent inducers of tissue factor (TF) expression in vitro but only on subsets of blood endothelial cells in vivo. Inflammation stimulation of TF on the inner layer of blood vessels results from the innate immune system and persistent, local vessel occlusion by thrombus. Similar in lymphatics, even in the absence of platelets, repeated intradermal injections of TNFα induced dot formation within draining collectors but only at discrete locations (Fig. 1C). No cumulative effect was observed when TNFα was co-injected with IL-1β, and this treatment produced a similar sparse distribution of clots within collectors. Activation of G-protein coupled receptor (GPR) 18 on inflammatory cells only removes the endothelial source of anti-thrombotic factors (thrombomodulin, plasminogen activators) but also expose negatively charged basement membranes, which laminin and collagen I can activate intrinsic coagulation cascade.
mix, lymph clots did not form within lymphatic collectors, and TF expression was reduced or absent on lymphatic endothelial cells. Arrows point to fibrin clots, arrowheads, MHCII-positive antigen presenting cells. L marks lymphatic collectors. TF intensity was normalized to the TF expression level of intestinal cells. Note that lymph clots are autofluorescent in the red channel due to cross-reactivity of anti-rat antibody with clot-entrapped mouse IgGs, hence the TF signal can be appreciated only at the edges or outside the clot. Scale bar: 50 µm.

sufficient to induce clot formation. Inhibition of anti-clotting pathway by thrombomodulin blocking leads to instant dissipated intralymphatic lymph clotting. There is a low basal level of tissue factor-dependent activation or ‘idiing’ of the clotting system in normal individuals resulting in a constant, low level of formation of thrombin. In the absence of an anti-clotting mechanism, the activation threshold of clotting cascade is crossed, and clotting cascade is initiated. The necessity of co-injection of mixed active and inactive thrombin to initiate lymph clotting suggested that inactivated thrombin served a role in activation of lymph clotting. Thrombin has two types of receptors on endothelial cells, thrombomodulin and protease-activated G-coupled receptors (PAR-1 on endothelium). PAR-1, however, has to be activated by thrombin proteolytic cleavage, hence inactivated thrombin cannot initiate its signaling. On the contrary, thrombin binding by thrombomodulin is independent on its enzymatic state when inhibited by small molecular inhibitors at thrombin active site. Thrombomodulin-bound thrombin shifts its enzymatic specificity from fibrinogen to protein C, which activation, in turn, leads to the destruction of co-factors V and VIII and inhibition of clotting process. Hence, blocking thrombin binding sites on thrombomodulin can inhibit the whole anti-clotting pathway. Treatment of thrombin with the potent, small-molecule covalent inhibitor p-(amidomphenyl) methane sulfon fluoride (APMSF) inactivates 99.75% of the enzyme and in our hands, it prolonged 400- times the clotting time. A single injection of saline had no effect on lymph clotting or dextran drainage (Fig 3A).

Figure 2. Intralymphatic coagulation induced by cytokine or partially inactivated thrombin is dependent on tissue factor expression on lymphatic endothelial cells. A, Schematic of a confocal whole-mount image of lymphatic collectors occluded by intralymphatic clot after two consecutive intradermal injections with pro-clotting factors with isotype control or tissue factor blocking antibody (21E10). Basement membrane (BM) was stained by collagen IV, antigen-presenting cells were stained with MHCII. A, Injection of partially inactivated (thrombinac) together with isotype control antibody induced high expression of TF on discrete dot-like location of collectors. Locations of lymph clotting coincided with high expression of tissue factor (TF) on collector endothelium (red within collector’s vessels). Collectors that express a high level of TF are densely infiltrated with MHCII-positive antigen presenting cells. B, Similar, in the presence of the isotype control antibody, mix of partially inactivated thrombin (thrombinac) and cytokines (TNFa and IL1B) induced fibrin clot formation in discrete locations within downstream collecting vasculature. In contrast to thrombinac alone, these clots were not infiltrated with MHCII-positive leukocytes. When αTF antibody was injected with the pro-clotting factor, fibrin clots were formed at sites of injection (*). Scale bar: 500 µm, 50 µm.

Figure 3. Completely inactivated thrombin blocks lymphatic drainage. A, Schematic of experimental design. Intradermal injection of saline, active thrombin (thrombin+, or completely inactivated thrombin (thrombinac) was followed by intravenous injection of fluorescently labeled fibrinogen. The mouse is fixed by perfusion 13 hours later. B, Top, Lymphangiography and intra-lymphatic fibrin in collectors of live tissue 5 hours after intradermal injection. Bottom, Imaging of fluorescent insoluble fibrin clot in fixed perfused dorsal skin flap after carriage and ventral skin were removed. Two adjacent injections of 2.5 µl PBS had no effect on the collector-drainage later (PBS, top). C, Fibrin formed only in the interstitium at the injection sites (PBS, bottom). Active thrombin (2.5 IU; thrombin+) increased hyperpermeability of collectors but did not block their drainage (Top, thrombin+). D, Fibrin formed within blood vessels around the site of injection (Bottom, thrombin+). In contrast to thrombin-, intradermal injection of inactive thrombin (thrombinac) occluded remote lymphatic collector vessels (Top, thrombinac). Drainage discontinuities visible on lymphatic lymphangiography are bridged by green fibrin deposits, indicating an incomplete occlusion. In contrast to continuous fibrin clots induced in blood vessels by thrombin+, thrombinac induced the formation of discontinuous clots in a large number of remote lymphatic collectors but had no effect on plasma coagulation in blood vessels (Bottom thrombin+). C, Higher magnification confocal images 5 hours after the second injection of compounds indicated in B. Collagen IV stained lymphatic collectors (L) and blood vessels (B) identified by their characteristic morphology (valves, uneven diameter along the vessel length). In PBS injected mice, fibrin clots were not formed away from the injection site. CD31 showed normal cell-cell junctions between lymphatics (PBS). Active thrombin blocked blood circulation, trapping autofluorescent erythrocytes (yellow) within blood vessels (B) around the injection site. Rare intralymphatic clots (L) could be found only around the thrombin+ injection site (Thrombin+). In the Thrombin+ injected skin, disrupted fibrin clots in remote lymphatic collectors were observed. D, Top, 15 hours after injection of PBS, tissue plasminogen activator (TPA) is randomly distributed within the skin but is also stain morphologically distinct macrophages (M), erythrocytes trapped within blood vessels, TPA-positive macrophages but also large vessels were seen around the site of Thrombin+ injection. In the skin injected with Thrombin+, TPA strongly labeled collecting lymphatic vessels filled with residual, centrally-located fibrin clots. Bottom, Imaging of the same field stained with collagen IV depicted tissue stationary structures, i.e., blood vessels, lymphatics, and adipocytes. A-Adipocytes, L-lymphatic collectors, B-blood vessels, M-macrophages, H-fallopian. The intensity of TPA was normalized to its signal level in interstitial cells. The intensity of lymphatic fibrin deposits was normalized to signal level of clots at the sites of injection (*). Scale bar: B–500 µm, C-50 µm.

Figure 4. Schematics of experimental design. Figure 3A–C: Drainage discontinuities visible on lymphatic lymphangiography are bridged by green fibrin deposits, indicating an incomplete occlusion. In contrast to continuous fibrin clots induced in blood vessels by thrombin+, thrombinac induced the formation of discontinuous clots in a large number of remote lymphatic collectors but had no effect on plasma coagulation in blood vessels (Bottom thrombin+). C, Higher magnification confocal images 5 hours after the second injection of compounds indicated in B. Active thrombin blocked blood circulation, trapping autofluorescent erythrocytes (yellow) within blood vessels (B) around the injection site. Rare intralymphatic clots (L) could be found only around the thrombin+ injection site (Thrombin+). In the Thrombin+ injected skin, disrupted fibrin clots in remote lymphatic collectors were observed. D, Top, 15 hours after injection of PBS, tissue plasminogen activator (TPA) is randomly distributed within the skin but is also stain morphologically distinct macrophages (M), erythrocytes trapped within blood vessels, TPA-positive macrophages but also large vessels were seen around the site of Thrombin+ injection. In the skin injected with Thrombin+, TPA strongly labeled collecting lymphatic vessels filled with residual, centrally-located fibrin clots. Bottom, Imaging of the same field stained with collagen IV depicted tissue stationary structures, i.e., blood vessels, lymphatics, and adipocytes. A-Adipocytes, L-lymphatic collectors, B-blood vessels, M-macrophages, H-fallopian. The intensity of TPA was normalized to its signal level in interstitial cells. The intensity of lymphatic fibrin deposits was normalized to signal level of clots at the sites of injection (*). Scale bar: B–500 µm, C-50 µm.
around the site of the injection together with blood vessel clots entrapping autolysed erythrocytes within blood vessels (Fig. 3C). Fast dissolution of intralymphatic clots (after injection of inactive thrombin, thrombin-in/ac) could be explained by increased expression of tissue plasminogen activator by lymphatic endothelial cells. Indeed, 16 hours after injection of thrombin-in/ac, lymphatic collecting vessels emerged from other tissue structures by an elevated level of TPA expression (Fig. 3D). Lymphatic vessels (thrombin-in/ac) at the site of injection-induced larger tissue damage and robust activation of TPA in various tissue cells, i.e., adipocytes, macrophages and also blood vessels, making these structures to stand out from surrounding tissue. In regular non stimulated tissue TPA was mostly expressed by macrophages, but with the exception of hair follicles, no stationary structures could be identified by distinctive TPA expression. A similar mechanism might be responsible for fast clearance of lymph clots in inflammatory factor-treated lymphatics where we also observed a high level of TPA expression within stimulated lymphatics (not shown). Intradermal clot deposited in the skin outside blood vascular reman stays within the skin in roughly unchanged shape for at least 24 hours (Fig. 4A). In contrast, thrombin-in/ac-induced blood vessel clots were almost entirely cleared from the site within 16 hours (Fig. 4B). The thrombomodulin blockage lost its dependency on TF after tissue pre-injection with TF-blocking antibodies. This possibility was excluded as we showed that mock pre-injection or injection with TF-blocking antibodies and subsequent injection of skin with TF-blocking antibodies and thrombin-in/ac did not inhibit the circulation. Re-injection of saline and thrombin-in/ac re-created clots in dermis and blood vasculature, respectively.

...leads to localized sterilir inflammation that should have multifactorial pro-hemostatic effects on the transported lymph. First, the elimination of thrombomodulin anti-clotting mechanism induced to lymphatic endothelial cells promotes amplification of contact-dependent pathway on exposed RNA and polynucleotides from dying cells. Also, decelerization of the lymphatic surface should prolong the occlusion of lymphatics as it eliminates the source of endothelium-derived tissue plasminogen activator that is a cause of the fast clearance of intralymphatic clots from TF-expressing or thrombomodulin blocked lymphatics (Fig. 3E). Finally, sterile inflammation leads to infiltration of TF-expressing hydroyl and hydroperoxyl radicals from mitochondria-derived hydrogen peroxide and it is used in TF-independent blood vessel thrombus formation models. Intradermally injected clots maintained their lumen: clot occlusion is not due to destruction of endothelial cells but due to the TF-expressing macrophages present in the lymphatic endothelium. In contrast, recurrent injection of saline activates an inflammation that leads to endothelial cell apoptosis (50) within the lymphatic endothelium (Fig. 3D). In these conditions both clots were normally dissolved after 16 hours.
that blocking of only a few lymphatic collectors ceases the drainage from the whole lymphatic tree. Including intact vessels draining the lateral side of the ear skin which are connected to the occluded lymphatic (Fig. 5D and F). This approach unselectively exerts bystander toxicity to any cell type present at the site of injection. Also, toxins cannot be inactivated and instead they can freely diffuse outside the collectors to potentially injuring cells in remote locations. Instead, photodynamic therapy (PDT) offers a solution to all these limitations. PDT is a combined physicochemical treatment where intradermally injected photosensitizer acts as proto-toxin, which is activated and destroyed with laser light at a defined location. This treatment assures that the toxicity of free radicals diffusing on short distances (up to 7 µm) will be strictly limited to collectors’ associated cells8. Large, 100nm in diameter Visudyne® liposomes enclose an active compound, a small molecular verteporfin, therefore their diffusion potential in the tissue was minimal. Practically, Visudyne® liposomes were either directly injected to the lymphatic collectors or retain in the skin interstitium. To avoid direct toxicity to the dermis that could result in enhanced trafficking of immune cells from the afferent injury site, the injection spots were painted black and covered with foil immediately after the injection only leaving the exposed collectors. We reached 98% of the reproductibility of the anti-lymphatic treatment by confirming phototoxic self-annihilation of Visudyne® within lymphatic collectors. This was done by imaging Visudyne® fluorescence before and after irradiation, which allowed us to eliminate mice with incompletely or poorly injected collectors. The routine anti-lymphatic treatment occluded all collectors draining peripheral dorsal skin within 24 hours after PDT with continuous lymph clots (Fig. 6B). Likewise injection of directly-acting toxins, Triton or ferric chloride (Fig. 5), and in contrast to TF-stimulation (Fig. 1-2) or thrombomodulin blocking (Fig. 3-4), PDT always resulted in the formation of continuous intralymphatic fibrin clots in all vessels that drained photosensitizer (Fig. 6B). In contrast to directly-acting toxins, PDT resulted in much lower collateral damage to the tissue at the injection site that was manifested in minimal leakage of fluorescent plasma into a tissue (compare Fig 5C and D with Fig. 6B). Endothelial cell death deduced from degeneration of cellular junctions was specific to endothelial lining in direct contact with a photosensitizer, leaving afferent vessels draining lateral side of ear dermis intact (Fig. 6D). Basement membrane that supports endothelium and muscle cells of lymphatic collectors (Fig. 6D), remained as a scaffold

**Figure 6. Specific decellularization of lymphatic collectors leads to complete and long-lasting lumen occlusion.** A. Schematics of experimental design. Endothelium was stripped from lymphatic collectors with a photodynamic therapy (PDT). Specifically, intradermal (i.d.) injection of photosensitizer verteporfin Visudyne® was followed by collector imaging (verteporfin lymphangiography) and subsequent irradiation of dorsal ear skin. 2D-30 min after PDT, fluorescently labeled fibrinogen was injected intravenously, and ear lymphatics were imaged the following day. The mouse was fixed/perfused on the indicated day for whole mount imaging. B. Intralymphatic imaging of whole-mount skin preparations. C. Left: SC injection and repeated lymphangiography had no effect on collector perfusion in the control ear. Middle: PDT leads to occlusion of lymphatic collectors, which stop draining i.d. injected fluorescent dextran. Right: The same collector networks become occluded with
in insoluble fibrin that leaked from blood vessels. C-E. Whole-mount imaging. Left: VE-cadherin is localized at cell-cell junctions in regular lymphatic collectors of the ear skin. Middle, Right: VE-cadherin organization is lost in vessels that drained photosensitizer (Vert), while in the lymphatics draining lateral to the Visudyne® treatment, clonal collections and vessel-like structures (5 µm away) were not affected by irradiation. D. Left: Lymphatic endothelial cells (VE-Cad-VE-cadherin) were enclosed within the basement membrane (stained for laminin, P-basement membrane and vessel pericytes). Middle: Morphologically unaffected, cell-deprived basement membrane tubes-ghost vessels’- one day after PDT. BM scaffold was preserved in PTD-decellularized collectors. Right: Ghost vessels were filled with fibrin clots that blocked the whole lymphatic lumen. Insert shows cross-section of the fibrin-oclcluded collector. Frequently, basement membrane particles could be found trapped within a clot (arrowhead). E. In addition to intralymphatic lymph clotting, ghost vessels became infiltrated with TGFβ1 leukocytes that firmly pack within the collector. F. From day 4 to 7 fibrin clots are cleared and partially replaced with tissue deposits containing basement membrane elements of laminin that are subsequently removed from vessel lumen (day 7 to day 10). The intensity of CD31 was normalized to its signal level in the lymphatics draining control region. The intensity of lymphatic fibrin deposits was normalized to the signal level of clots at the sites of injection (*). Scale bar: B, 250µm, C, D, E-100µm, F-25µm.

In contrast to lymph clotting provoked by cytokine-activated TF-expression, intralymphatic clotting after PTD-fibrinolysis could not be prevented with TF blocking. The intensity of fibrin deposits was normalized to the signal level of clots at the sites of injection (*). Scale bar: B, 250µm, C, D, E-100µm, F-25µm.

Video 2. Concurrent necrosis and apoptosis of B16-F10 cells. Video 1. Lymphatic endothelium died off within two hours after photodynamic therapy. The video presents a higher temporal resolution of events shown in figure 7A. Morphology of major collectors was identified with intralymphatic staining for basement membrane (collagen IV) and co-expression of Tomato protein. For clarity, collagen IV staining is not shown on the video (see Fig. 7A). As images were taken every one minute, Tomato-positive cells did not earlier than 1.5 hours appear in the visualization. The real-life duration of the video is 2 hours. The video frame rate is 15 fps. Video 2. Concurrent necrosis and apoptosis of B16-F10 cells grown for one week in the mouse ear. Single cell divided but the two daughter cells die by apoptosis (top cell breaks into small apoptotic bodies which intensity is not different to the mother cell) and necrosis (bottom cell “explode,” the GFP fluorescence diffuse out in 1-2 frames of the 15 fps movie). The color gradient, from high-contrast white to green, representing the weakest signal was chosen for the clarity of the visualization. The real-life duration of the video is 2 hours. The video frame rate is 15 fps.

The majority of endothelial cells treated with PTD diffuse their cytoplasmic proteins, the more informative indicator of the final stage of necrotic cell death (Suppl video 3). Despite that collector’s endothelium dies off within 2.5 hours after PDT vessel occlusion started not earlier than 3 hours after PDT (Fig. 7B), which is in agreement with our previous results49. Here we show that necrotic death of lymphatic endothelial cells at the level of the valve 2 hours after photodynamic therapy. Propidium iodide, a membrane impermeable DNA-binding agent labels the valve 2 hours after photodynamic therapy. The video presents a higher temporal resolution of events shown in figure 7A. Morphology of major collectors was identified with intralymphatic staining for basement membrane (collagen IV) and co-expression of Tomato protein. For clarity, collagen IV staining is not shown on the video (see Fig. 7A). As images were taken every one minute, Tomato-positive cells did not earlier than 1.5 hours appear in the visualization. The real-life duration of the video is 2 hours. The video frame rate is 15 fps.

Massive infiltration of antigen presenting cells and T-cells begin after lymphatic occlusion and persist until their regeneration. Necrosis death of lymphatic endothelial cells at the level of the valvular 2 hours after photodynamic therapy. Propidium iodide, a membrane impermeable DNA-binding agent labels nuclei of necrotic cells. The real-life duration of the video is 6 hours. The video frame rate is 4 fps. We showed before that cell killing with PTD occurs by necrosis as cells become permeable to propidium iodide within 1.5 hours after the treatment44. Here we show that necrotic death of lymphatic endothelial cells at the level of the valve 2 hours after photodynamic therapy. Propidium iodide, a membrane impermeable DNA-binding agent labels nuclei of necrotic cells. The real-life duration of the video is 6 hours. The video frame rate is 4 fps. We showed before that cell killing with PTD occurs by necrosis as cells become permeable to propidium iodide within 1.5 hours after the treatment44.

Necrotic death of lymphatic endothelial cells at the level of the valve 2 hours after photodynamic therapy. Propidium iodide, a membrane impermeable DNA-binding agent labels nuclei of necrotic cells. The real-life duration of the video is 6 hours. The video frame rate is 4 fps. We showed before that cell killing with PTD occurs by necrosis as cells become permeable to propidium iodide within 1.5 hours after the treatment44. Here we show that necrotic death of lymphatic endothelial cells at the level of the valve 2 hours after photodynamic therapy. Propidium iodide, a membrane impermeable DNA-binding agent labels nuclei of necrotic cells. The real-life duration of the video is 6 hours. The video frame rate is 4 fps. We showed before that cell killing with PTD occurs by necrosis as cells become permeable to propidium iodide within 1.5 hours after the treatment44. Here we show that necrotic death of lymphatic endothelial cells at the level of the valve 2 hours after photodynamic therapy. Propidium iodide, a membrane impermeable DNA-binding agent labels nuclei of necrotic cells. The real-life duration of the video is 6 hours. The video frame rate is 4 fps. We showed before that cell killing with PTD occurs by necrosis as cells become permeable to propidium iodide within 1.5 hours after the treatment44. Here we show that necrotic death of lymphatic endothelial cells at the level of the valve 2 hours after photodynamic therapy. Propidium iodide, a membrane impermeable DNA-binding agent labels nuclei of necrotic cells. The real-life duration of the video is 6 hours. The video frame rate is 4 fps. We showed before that cell killing with PTD occurs by necrosis as cells become permeable to propidium iodide within 1.5 hours after the treatment44. Here we show that necrotic death of lymphatic endothelial cells at the level of the valve 2 hours after photodynamic therapy. Propidium iodide, a membrane impermeable DNA-binding agent labels nuclei of necrotic cells. The real-life duration of the video is 6 hours. The video frame rate is 4 fps. We showed before that cell killing with PTD occurs by necrosis as cells become permeable to propidium iodide within 1.5 hours after the treatment44. Here we show that necrotic death of lymphatic endothelial cells at the level of the valve 2 hours after photodynamic therapy. Propidium iodide, a membrane impermeable DNA-binding agent labels nuclei of necrotic cells. The real-life duration of the video is 6 hours. The video frame rate is 4 fps. We showed before that cell killing with PTD occurs by necrosis as cells become permeable to propidium iodide within 1.5 hours after the treatment44. Here we show that necrotic death of lymphatic endothelial cells at the level of the valve 2 hours after photodynamic therapy. Propidium iodide, a membrane impermeable DNA-binding agent labels nuclei of necrotic cells. The real-life duration of the video is 6 hours. The video frame rate is 4 fps. We showed before that cell killing with PTD occurs by necrosis as cells become permeable to propidium iodide within 1.5 hours after the treatment44. Here we show that necrotic death of lymphatic endothelial cells at the level of the valve 2 hours after photodynamic therapy. Propidium iodide, a membrane impermeable DNA-binding agent labels nuclei of necrotic cells. The real-life duration of the video is 6 hours. The video frame rate is 4 fps. We showed before that cell killing with PTD occurs by necrosis as cells become permeable to propidium iodide within 1.5 hours after the treatment44.

Necrotic death of lymphatic endothelial cells at the level of the valve 2 hours after photodynamic therapy. Propidium iodide, a membrane impermeable DNA-binding agent labels nuclei of necrotic cells. The real-life duration of the video is 6 hours. The video frame rate is 4 fps. We showed before that cell killing with PTD occurs by necrosis as cells become permeable to propidium iodide within 1.5 hours after the treatment44. Here we show that necrotic death of lymphatic endothelial cells at the level of the valve 2 hours after photodynamic therapy. Propidium iodide, a membrane impermeable DNA-binding agent labels nuclei of necrotic cells. The real-life duration of the video is 6 hours. The video frame rate is 4 fps. We showed before that cell killing with PTD occurs by necrosis as cells become permeable to propidium iodide within 1.5 hours after the treatment44. Here we show that necrotic death of lymphatic endothelial cells at the level of the valve 2 hours after photodynamic therapy. Propidium iodide, a membrane impermeable DNA-binding agent labels nuclei of necrotic cells. The real-life duration of the video is 6 hours. The video frame rate is 4 fps. We showed before that cell killing with PTD occurs by necrosis as cells become permeable to propidium iodide within 1.5 hours after the treatment44.
of PDT-treated lymphatics, which was confirmed with that only a few IgG-expressing cells were present in the tissue. IgGs derived from blood plasma are also trapped within +2 days class II-positive antigen presenting cells. While Gr1-positive neutrophils and monocytes remained in the surrounding dermis. persists for the whole regeneration time of lymphatic, observed up to 10 days. The majority of cells within lymphatics are MHC staining. Arrows point to leukocytes clusters within ghost vessel. Arrowheads point to intralymphatic protein clusters visualized with anti-mouse IgG antibody used to reveal mouse anti-αSMA lymphatics become densely filled with leukocytes making this leukocyte manifestation an identifier for decellularized vessels. A change in leukocyte accumulation within lymphatics occurs between 24 and 48 hours after PDT-dependent decellularization - vessel decellularization, although leukocyte build-up in peri-lymphatic tissue is already apparent at 6 hours after PDT. A dramatic Leukocyte accumulation within ghost vessels cannot be distinguished from that of control lymphatics for the first 24 hours after decellularization. Arrowheads point to the border of a lymphatic collector marked by a residual sheet of fibrin, which delineates characteristic uneven diameter along the length morphology of lymphatic collecting vessels. Scale bar: 50µm.

Figure 8. Massive infiltration of antigen presenting cells and T-cells into ghost vessels follows intravascular lymph clotting. A. Time course of leukocyte (CD45+ cell) infiltration into ghost vessels (as visualized by staining of laminin of basement membrane) leukocyte accumulation within ghost vessels cannot be distinguished from that of control lymphatics for the first 24 hours after vessel decellularization, although leukocyte build-up in peri-lymphatic tissue is already apparent at 6 hours after PDT. A dramatic change in leukocyte accumulation within lymphatics occurs between 24 and 48 hours after PDT-dependent decellularization - lymphocytes become densely filled with leukocytes making this leukocyte manifestion an identifier for decellularized vessels. Arrowheads point to intralymphatic protein clusters visualized with anti-mouse IgG antibody used to reveal mouse anti-αSMA staining. Arrows point to leukocytes clusters within ghost vessel. B. Dense leukocyte accumulation within lymphatic vessels persists for the whole regeneration time of lymphatic, observed up to 10 days. The majority of cells within lymphatics are MHC class II-positive antigen presenting cells. While Gr1-positive neutrophils and monocytes remained in the surrounding dermis. C. 2 days after decellularization. Single imaging field showing F4/80- neutrophils and macrophages infiltrating dermis-surrounding collectors. However, they almost exclusively remain in the tissue and do not invade ghost vessels. 4 days after decellularization. Single imaging field is showing that Gr1+ T-cells are the other abundant cell type that swarms ghost vessels by 4 days after decellularization. Arrowheads point to the border of a lymphatic collector marked by a residual sheet of fibrin, which delineates characteristic uneven diameter along the length morphology of lymphatic collecting vessels. Scale bar: 50µm.

IgGs derived from blood plasma are also trapped within anti-B2B staining (not shown). The delayed infiltration of collectors is probably due to the preservation of afferent initial lymphatics and injection site, which in case of PDT-treated lymphatics, which was confirmed with as one necrotic zone (Fig. 5). This also indicated that it is fibrin clotting and not leukocyte accumulation within decellularized lymphatic directly followed endothelial cell death and was accurately correlated with lymphatic occlusion. Interestingly, the antigen presenting cells (Fig. 8B-C) and T cells consist of the vast majority of cells that infiltrated ghost vessels while Gr1-positive neutrophils or monocytes or F4/80-positive macrophages and monocytes only populated ghost vessels-surrounding dermis (Fig. 8C). Antigen-presenting cells that populated ghost vessels expressed CD11c (Supplementary Fig. 4), the marker of dendritic cells. The presence of endothelium on collectors is not necessary for fluid drainage and cell trafficking. Decellularization of collectors leaves an intact tube of basement membrane that is initially filled with impermeable fibrin clots to be later replaced by the basement membrane-like matrix (Fig. 6). Since these ghost vessels were capable for at least short time drainage after decellularization, we asked whether lymphatic endothelium is at all needed for fluid drainage and traffic leukocytes to the lymph nodes. To do that, we tried to block lymph clotting by intradermal
organ. Allografts are accepted indefinitely when that connects the drained skin with secondary lymphoid skin transplantations confined to autografting. However, the effect of the immunosuppression on allograft tolerance, due to the potent and acute cytotoxic response and little detection of the immune system, is sufficient to hide allograft from lymphatic occlusion alone does not produce lymphedema. Absence of persistent inflammation lymphatic occlusion, however, in agreement with early observations, wherein the results prove that occlusion of collectors with clotted lymph is sufficient to completely block the drainage of macromolecules (e.g., antigens) and trafficking of any kind of cells from the tissue to the draining lymph node. Additionally, skin edema that develops one day after PDT was resolved on day 5 before any functional regeneration of lymphatics took place. This is however in agreement with early observations, wherein the absence of persistent inflammation lymphatic occlusion alone does not produce lymphedema.

**Lymphatic occlusion is sufficient to hide allograft from the detection of the immune system**

Due to the potent and acute cytotoxic response and little effect of the immunosuppression on allograft tolerance, skin transplantations confined to autografting. However, cutaneous immunity has one significant vulnerability as its responsiveness depends on functional lymphatic system, that connects the draining skin and secondary lymphoid organ. Allografts are accepted indefinitely when transplanted to a location that could not drain to the lymph nodes because of induction of immune ignorance rather than tolerance. Since we understood the occlusion mechanism and the kinetics of lymphatic regeneration, next, we planned to test the efficacy of the temporal lymphatic blockage on the prolongation of subcutaneous allograft acceptance. To do that we established highly reproducible and sensitive subcutaneous heart transplant model. In contrast to skin-over-skin transplant assays, transplantation of cardiac tissue was transplanted under ear dermis of the fully immunocompetent mouse with or without lymphatic-specific photodynamic therapy (PDT) and observed for functional muscle contraction, plotted as % graft acceptance rate vs. number of days of graft survival. Allografts transplanted under control mouse dermis, the time of allograft acceptance was almost doubled from the initial vascularization of the transplants (over 95%, Fig. 10A), which is the most critical parameter in probing immune ignorance of the initial vascularization of the transplants (over 95%, Fig. 10A), which is the most critical parameter in probing immune ignorance of the rejection kinetics in control and PDT groups, were different. While the control tissues were rejected across the 5 day period, the rejection in PDT group is almost binominal and occurred within one day. This could be explained by the high level of lymphatic regeneration homogeneity, but also the fact that ghost vessels before the regeneration of lymphatics become tertiary lymphoid organs, with an accumulation of antigen presenting cells ready to be delivered to the draining lymph node (Fig. 8). Conceivably, as the time of vessels regeneration was delayed with anti-VEGFR3 therapy (Fig. 10C), the time of allograft acceptance was almost doubled from the original 6 to 15 days. Interestingly, this value is better than presented with the same assay results on the effect of commercial cyclosporine (Sandimmune) treatment on allograft rejection that occurred after 9 days in control and 13 days cyclosporine-treated group. Alloantigen-specific antibody that develops after the tissue implantation is generally considered not relevant for an acute cytotoxic response. Yet, their level and subtype constitute significant parameter in probing immune ignorance as the production of allo-specific antibody, and dorsal skin completely protected the implant from drying and infection and provided blood supply from the uninjured dorsal dermis. This assured a high level of the initial vascularization of the transplants (over 95%, Fig. 10A), which is the most critical parameter in avoidance of artifacts like considering necrotic rejection as immunological rejection. Transplanted under dorsal ear skin halves of the newborn mice hearts were vascularized within 5 days after implantation and started contraction that was monitored through the skin (Supplementary video 5).
and antibody class switching reflects the availability of alloantigens in the lymph node. The seven-fold relative increase in IgM alloantibody was the highest between all tested isotypes (Fig. 10C) indicating that anti-lymphatic (PDT) therapy reduced the increase of allospecific antibody to 1.4 fold (2.4-times), with further reduction to the basal level (0.4 fold increase) was observed when PDT was followed by lymphatic regeneration delay with αVEGFR3 therapy. Even though the level of antibody in PDT and PDT-αVEGFR3 treated groups were not different to the respective basal levels, the pattern of antibody decline was not different. Experiments with injection of necrotizing compounds, i.e. TritonX and FeCl3 (Fig. 5) aimed to confirm previous observation where intradermally administered turpentine or FeCl3,40 led to cessation of lymphatic drainage by intralymphatic injection of hydrogen peroxide (‘thrombosis’, experiments reviewed by Drinker and Field41).

Both, TritonX and ferric chloride exerted toxicity at the site of injection but also completely blocked the skin drainage and induced lymph clotting in the lymphatics located remotely to the site of intradermal injection. The same effect of lymphatic occlusion but without adverse skin, nesocis was achieved with PDT-induced lymphatic decellularization (Fig. 6-10). Due to the high specificity of anti-lymphatic treatment and the long-lasting (9 days) drainage occlusion, we used this method to study the effects of intravascular lymphatic clotting on lymphatic physiology and local immune responses. Despite the type of lymphatic treatment, lymph clotting was an endpoint that preceded interference or complete blockage of fluid drainage leakage rates involvement in fibrin polymerization and maturation within lymphatics could not be excluded; the initial lymph coagulation occurred before leukocytes infiltration (Fig. 2 and 8). Therefore, in one way or another lymph clotting does not have a leading role. Evidence that either expressed TF after stimulation lost anti-hemostatic properties after thrombomodulin blocking or was destroyed by detergent lysis (Triton-X-100), exposure to free radicals generated by FeCl3, or free radicals generated by light-activated verteporfin (i.e., lymphatic-specific PDT). However, the duration of lymphatic occlusion varies significantly between the metabolic and noxious methods of lymph clotting, from less than 22 hours with preserved intact endothelium to 9 days,45 when collectors were decellularized. Initial lymph clotting could be activated by dying lymphocytes releasing pro-inflammatory cytokines, RNA and polyphosphates that can activate factor XII of the intrinsic pathway can be also continuously activated by dying cells releasing pro-thrombotic negatively charged phospholipids and outside-in cell signaling. Therefore, the lack of conventional lymphatic drainage is a necessary but not sufficient factor of lymphedema, all causative factors are not known as lymphatic occlusion is a necessary but not sufficient factor of the disease. Filariasis-induced lymphedema develops after repeated instances of worm death and bacterial infections. Infections are not causative factors, but even after PDT-induced lymphatic decellularization, lymphatic regeneration is hampered and experimental infection with Leishmania major results in a delayed formation of inflammatory response.

However, the importance of anti-lymphatic therapy is limited to extemporary, e.g., therapeutic relevance is limited to extemporary, e.g., adjuvant therapies, e.g., by reinforcing clotted lymph with fibrinogen or by blocking the lymphatic occlusion with heparin (Fig. 9). Even though decellularized collecting lymphatics were leaky to drained exudate, this experiments indicated that endothelial lining is not indispensable for the most affirmed functions of lymphatics, the fluid drainage, and cell trafficking through PDT-decellularized collectors. Instead, lymphatic endothelium is primarily needed to support collector's patency. Intravascular lymph clots delay of allograft rejection -therapeutic applications

The lack of conventional lymphatic drainage is a characteristic feature shared between passive immune injury and immune privilege at sites like placenta, cheek pouch, cornea or central nervous system.66 Furthermore, it was shown that surgical separation of draining lymphatics blunts the draining lymph nodes to antigens residing within the blocked area and prevent the pathogenesis of an immune response.67 On the other hand, the ability of dysfunction of lymphatics to sustain non-clotted lymph might contribute to their pathologies with idiopathic or confound origin. Surprisingly, even in case of thoroughly studied lymphedema, all causative factors are still insufficient factor to induce sustained lymphedema in animal models.68, 69. Also, lymph clots within lymphatics around the granuloma that form after worm death during the pathogenesis of filarial lymphedema intuitively carry a notion that lymph clotting and inflammation might be important priming factors of the disease. Filaria-induced lymphedema develops after repeated instances of worm deaths and bacterial infections that additionally lead to lymphatic endothelial injury and fibrotic remodeling of lymphatic collectors.68 Along these lines, our recent indicated that inflammatory factors-stimulated lymphatic occlusion is transient and quickly resolved within lymphatic vessels, while injury to lymphatic endothelial lining is sufficient for prolonging occlusion of the collector. But even after PDT-induced lymphatic decellularization, the ghost vessel eventually regenerated instead of becoming fibrotic.68 PDT-decellularization is a specific and sterile anti-lymphatic treatment while trauma combined with infectious inflammation might produce long-lasting occlusion of lymphatics,69 which translates to further sclerosis of lymphatic collectors with collagenous fibrosis, elements of which we could observe in lymphatics regenerating after PDT (Fig 6F). Also, anti-hemostatic properties of lymphatics should be the primary parameter that requires

lymphangiographic images showing drainage at day 11 after PDT in PDT and PDT+αVEGFR3 ears and the drainage in the same PDT+αVEGFR3 ear on day 17. Arrows show the direction of regenerated drainage. Arrowheads on day 11 and day 17 point to the same lymph node (a 250μM laser microminiature of complement phagocytes-activating alloantibodies (cell-dependent cytotoxicity, CDC) of all classes in the sera of mice in control, PDT and PDT+αVEGFR3 groups, respectively. Experimental control is shown as a point of reference. Bars indicate the difference between groups tested with ANOVA and Holm-Sidak post-priori test at p<0.05.
consideration before designing lymphatic implants, e.g., artificial collectors lympho-venous shunts or lymphatic collateral neovessels26 as they must anchor functional anti-
hemostatic fluids that mimic lymphatic tissue permanently. Because until now, the anti-hemostatic
property of lymphatic has not been yet fully appreciated, it is possible that lymph cloting and subsequent fibrosis is an underestimated complication unwittingly hampering lymphatic treatments. For example, unpredictable clotting and fibrosition cycles could explain the fact that most silicon tubes implanted in lymphedema patients did not drain the fluid along their lumen even though it did nor undergo fibrosis and remained patent27.

The active regulation of lymphatic drainage might also affect tumor metastasis. Entrapped tumors or associated cells might activate fibrinolytic mechanisms and use residual fibrin scaffold to enhance their migration within the lymphatic system. This scenario is supported by experiments where depletion of fibrinogen inhibits lymphatic metastasis but has no effect on primary tumor growth26. Contrarily, completely occluded lymphatics might stop the spread of metastatic cells, likely the mechanism of PDT treatment that blocked the metastasis spread via peri-tumoral lymphatics 4. In this situation lymphatic cloting and fibrin deposition is a potential new avenue for the research that could, e.g. aim at reinforcement of fibrin or local inhibition of fibrinolysis4 in order to prolong a block cell dissipation along lymphatic collectors. Strengthening of intralymphatic clot resilience could also be the key factor in restoration of lymphatics occluded with PDT and by this extend the time subcutaneous allografts are masked and ignored by the immune system. Accessibility of the dermal lymphatics and low-risk subcutaneous surgery might permit therapeutic prolytic treatment allowing the in situ transforming various tissue fragments or cells, like Langerhans islets 83.

Methods

Animal experiments

Animal experiments were performed at the EPFL (Lausanne, Switzerland), the University of Chicago and the Medical University of Vienna (tail edema experiments). Procedures performed on animals were in accordance with the Swiss Animal Protection Act, the ordinance on animal protection and the ordinance on animal experimentation. We confirm that our Institutional Animal Care and Use Committee (IACUC), named Commission de Surveillance de l’Etat de Vaud (Permit Number: 2646) approved this study or in accordance with protocols approved by the Institutional Animal Care and Use Committee at EPFL (Permit number 72414). Procedures performed at the University of Chicago were performed according to federal guidelines and protocols approved by the Institutional Animal Care and Use Committee at EPFL, the guidelines approved by the Ethics Committee of the Medical University of Vienna. BALB/c female or C57 mice were purchased from Jackson Laboratories or Charles River, maintained and bred in a specific pathogen-free barrier facility and used between 6 to 10 weeks of age unless stated otherwise.

Anesthesia and hair removal

Decapitation was performed with a vortexer (Milwaukee Milwaukee, WI, USA) equipped with 33 G needle. Splenocytes trafficking into lymphatics

Splenocytes from BALB/c mouse expressing eGFP under ubiquitin promoter were isolated from the mouse spleen by grounding the spleen through the strainer with 70µm pores. Red blood cells were lysed with RBC lysing solution (150 mM NH 4Cl and 10 mM Hepes) for 5 minutes, washed with PBS and resuspended in PBS. Triplicate samples were incubated with 25 µg/ml propyl gallate (reactive index 1.56). The tissue was mounted on a glass slide and image using HC PLAN 20x, NA 0.70 or HCX PL APO 63x, NA 1.40 lenses of a Leica SP5 confocal microscope (Leica). Stacks of images were analyzed with Imaris 7.4 (Bitplane AG).

Transplant experiments

PDT was performed on ears on day 0 and on the next day newborn (1-2 days after birth) mice hearts from allogenic or syngeneic mice were cut in half and incubated in DMEM medium. The incision was done on the ventral side of the mouse ear and spatica was inserted through the cartilage to incise the skin and separate the cartilage from dorsal ear skin. Inside the forming chamber, halve ear was inserted, and the ventral incision was sealed with surgical glue. 50 µl of the blood was collected on day 5 and 10, and isolated serum was frozen for further analysis. Heartbeat (in heart rate) was monitored for 1 day after short form ischemia. The beating stopped when mice were perfused, and their ears were zinc-ixed for further analysis.

Detection of allo-specific IgM and IgG in grafted mice

Thymocytes from the allogenic background were isolated from 4 weeks old mice at 10x10^7/ml. 20µl of cells were mixed with 5µl ofmouse serum isolated from mice bearing transplants on day 5 and 10. After two glass slides. For intravital lymphangiography, ventral skin was removed, and a frontal light distributor with a lens (Medlight) was inserted through a 1.5 mm incision. A Leica SP5 confocal microscope (Leica) was used for stacks of images were analyzed with Imaris (Bitplane AG).

Decellularization of lymphatics with detergent or FeCl3

Decellularized lymphatics were fixed in 4% formaldehyde for 2 hours, washed with PBS and then imaged using automated fluorescent stereomicroscope (M205 FA, Leica Microsystems) coupled to the sensitive color camera Leica DFC350 FX (Leica Microsystems). The following objectives were used: 4x, 10x, 20x and 40x. 0.25µl of 1000X or 600X or 500X or 300X or 200X or 100X or 50X or 10X or 5X or 2X or 1X, or 0.5µl of 1000X or 100X or 50X or 10X or 5X or 2X or 1X, or 0.25µl of 1000X or 100X or 50X or 10X or 5X or 2X or 1X, or 0.125µl of 1000X or 100X or 50X or 10X or 5X or 2X or 1X, or 0.0625µl of 1000X or 100X or 50X or 10X or 5X or 2X or 1X, for each day. The images were then combined into a single composite image using the software Leica Application Suite (LAS). The images were then exported and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).
References

1. Simbronne (1987). Vascular endothelium: Nature’s blood-compatible container. Am J Physiol.
2. Rossnak, L. (1987). Lymphatics and lymph circulation. Physiology and pathology. Pergamon Press.
3. Drinker, K. & al. (1933). Lymphatics, lymph and tissue fluid. The Williams & Wilkins Co.
4. Miller, R. (2000). Hormetic factors in human peripheral arterial thrombosis. Thromb Haemost.
5. Atwell (2006). Biochemical mechanisms in inflammation. In Motl I, ed.
6. Opie (1931). Thrombosis and occlusion of lymphatics. J Exp Med.
7. Copley (1942). Bleeding time, lymph time, and clot resistance in men. Arch Intern Med.
8. Ferrti, J. & al. (1953). Conjunctivitis in lymph. J Physiol.
9. Miyazaki, A. (1961). A comparative study of the cutting power of the blood and lymph. Bull Fac Med Sci.
10. Lea, C. & al. (1996). Hormetic factors in kidney. Lymph: Relationship to mechanisms regulating extravascular coagulation. Am J Physiol.
11. Leak, J. (2006). Protonic analysis of lymph. Protonomics.
12. Lips, L. & al. (2012). Hormetic properties of the lymph: Relationships with occlusion and thrombosis. Semin Thromb Hemost.
13. Brkinson, J. & al. (1996). Vessel wall-mediated activation of the blood coagulation system. Vascular control. Harwood Academic Publishers.
14. Drinker, K. (1933). Inflammation. Lymphatics, lymph and tissue fluid. The Williams & Wilkins Co.
15. Aukland, R. & al. (1993). Intravascular mechanisms in the control of extracellular fluid volume. Physiol Rev.
16. Wrig, J. & al. (2012). Intestinal fluid and lymph formation and transport: Physiological regulation and roles in inflammation and cancer. Physiol Rev.
17. Engberg, M. & al. (2013). Thrombin as an intravascular effector of innate immunity. Nat Rev Immunol.
18. Conway, T. (2012). Thrombomodulin and its role in inflammation. Semin Immunopathol.
19. Matsuyama, B. (1985). Thrombomodulin is found on endothelium of arteries, veins, capillaries, and lymphatics, and on syncytiotrophoblast. J Exp Med.
20. Kirchhoff, H. & al. (1994). Endothelial cells stimulated with tumor necrosis factor-alpha express varying amounts of tissue factor resulting in fibrinous intravascular debris in a native blood flow system. Effects of thrombin inhibitors. J Clin Invest.
21. Laschinger, L. & al. (1990). Production of plasminogen activator and plasminogen activator inhibitor by bovine lymphatic endothelial cells: Modulation by TNF-alpha. Thromb Res.
22. Drinker, K. & al. (1933). Lymph vessels and solphatics. Lymphatics, lymph and tissue fluid. The Williams & Wilkins Co.
23. Shamskii, M. & al. (2002). De novo lymph node formation in chronic inflammation of the human arm. Am J Surg.
24. Cara, F. (1991). Vascular abnormalities in experimental and human lymphatic hyperplasia. Lymphology.
25. Fader, A. & al. (1986). Evolution of lymph thrombus in experimental brugia malay infections. A scanning electron microscopic study. Lymphology.
26. Mara, C. & al. (2013). Lymphoedema caused by atypical lymphatic vessels. J Plast Reconstr Aesthet Surg.
27. Clusen (2007). Peripheral lymphedema treatment. Philipade.
28. Forstein, B. & al. (2006). Tissue factor and tumor. Clinical and laboratory aspects. Clin Cance.
29. Ru, B. & al. (2011). Tissue factor and cell signaling in cancer progression and Heremogens. J Thromb Haemost.
30. Paderna, L. & al. (2002). Lymphatic metastasis in the absence of functional intratumoral lymphatics. Science.
31. stil (2005). Coagulation, fibrinolysis, and fibrin deposition in acute lung injury. Crit Care Med.
32. Margaritescu, S. & al. (2015). Cutaneous metastases of breast carcinoma. Rare Tumors.
33. van der Meijden, F. & al. (2009). Dual role of collagen in factor x-dependent thrombin formation. Blood.
34. White-Adams, B. & al. (2010). Laminin promotes coagulation and thrombus formation in a factor x-dependent manner. J Thromb Haemost.
35. Beversloot, R. & al. (1994). Interleukin 2 (IL-2) induces synthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. J Exp Med.
36. Lipp, J. & al. (2005). Factor-dependent coagulation is preferentially up-regulated within arterial branching areas in a baboon model of escharic calcified vessel. Am Pathol.
37. Liu, B. & al. (2004). Thrombin and tumor necrosis factor alpha synergistically stimulate tissue factor expression in human endothelial cells: Regulation through a c-sis and c-jun. Blood.
38. Klarski, J. & al. (2013). Intratumoral immunofluorescence for visualizing the microvascular units and immune microenvironments in the mouse ear dermis. PLoS One.
39. Pinsky, M. & al. (2002). Endothelium-role in regulation of coagulation and inflammation. Semin Immunopathol.
40. Inba, T. & al. (2005). Proinflammatory cytokines of coagulation: Their role in threshold regulation. Arterioscler Thromb Vasc Biol.
41. Mackewn, S. (2009). The role of tissue factor and factor vixx in hemostasis. Annu Rev Anal.
42. Fantl, M. & al. (2000). Structural basis for the anticoagulant activity of the thrombin-thrombomodulin complex. Nat Rev.
43. Lu, B. & al. (2001). Sterile infection: Sensing and reacting to danger. Immunity.
44. Eskin (1970). For the heart: Countercurrent coagulation. Nat Med.
45. Wu, J. & al. (1996). Role of endothelium in thrombosis and hemostasis. Ann Rev Physiol.
46. Eppinger, T. & al. (2008). Granulocytes do not express but acquire monococyte-derived tissue factor in whole blood. Evidence for a direct transfer. Blood.
47. Wang, J. & al. (2005). Effects of factor x or factor x deficiency on fibrin cleavage-induced cardial artery occlusion in mice. J Thromb Haemost.
48. Klarski, J. & al. (2014). Optimization and regeneration kinetics of lymphatic-specific photodynamic therapy in the mouse dermis. Angiogenesis.
49. Inba, T. & al. (2004). Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. Am J Pathol.
50. van Kruisberen, M. & al. (2012). Regulation of endothelial cell plasticity by tgbf. Cell Tissue Res.
51. Guo, B. (2007). In vivo intraocular immunofluorescence imaging of tissue matrix components with epifluorescence and two-photon microscopy. J Vis Exp.
52. Randolph, J. & al. (2005). Dendritic-cell trafficking to lymph nodes through lymphatic vessels. Nat Rev Immunol.
53. Drinker, K. (1933). Relation of the lymphatics to edema and the consequences of lymphatic obstruction. Lymphatics, lymph and tissue fluid. The Williams & Wilkins Co.
54. Benshoof, J. (2011). Immune recognition and rejection of allogeneic skin grafts. Immunotherapy.
55. Lund, L. & al. (2000). Lymphatic vessels, inflammation, and immunity in skin cancer. Cancer Cell.
56. Lakki, B. & al. (2000). Immunogenic ‘ignorance’ of vascularized organ transplants in the absence of secondary lymphoid tissue. Nat Med.
57. Yamagami, K. (2001). The critical role of lymph nodes in cancer dissemination and graft rejection. Immunol. Immunobiol.
58. Fulmer, R. (1963). Transplantation of extracorporeal tissue into the mouse ear. J Exp Med.
59. McTighe, J. (2009). Skin allograft rejection.Curr Protoc Immunol.
60. Rudide, L. (2014). Lymphatic vessels and lymphatic organs. Tissue Immunol.
61. Geddie, L. (1991). Evidence that stromal incorporation of cytoskeletal responders increases its toxicity and potentiates its ability to prolong survival of cardiac allografts in mice. Transplantation.
62. Colvin, R. (2005). Antibody-mediatad organ-allograft rejection. Nat Rev Immunol.
63. Menkin, J. (1991). Studies on inflammation: V. Fixation of bacteria and of particulate matter at the site of inflammation. J Exp Med.
64. Menkin, J. (1931). Studies on inflammation: V. Fixation of trypan blue in inflamed areas of frogs. J Exp Med.
65. Menkin, J. (1931). Studies on inflammation: V. The mechanism of fixation by the inflammatory reaction. J Exp Med.
66. Menkin, J. (1931). An inflammation - a protective mechanism. Arch Pathol Sci Med.
67. Michael, E. & al. (2006). Lymphatic remodeling and contractile dysfunction in skin disease processes. Microcirculation.
68. Perls, J. & al. (1991). Lymphatic mapping and lymphedema surgery in the breast cancer patient. J Surg Oncol.
69. Olszewski, J. & al. (2015). A new method of external fluid drainage in obstructive lymphedema of limbs by implantation of hydraulic silicone tubes. Lasers Surg Med.
70. Palumbo, L. (2002). Intratumoral lymphangiogenesis and lymphatic metastasis, but not primary tumor growth or angiogenesis, is diminished in fibrillin-deficient mice. Cancer Res.
71. Tammele, A. & al. (2011). Photodynamic ablative of lymphatic vessels and intratumoral cancer cells prevents metastasis. Nat Protoc.
72. Pepper, R. (2015). A prosesuvalized subcutaneous device-less site for silt and cellular transplantation. Nat Biotechnol.
Supplementary Fig. 1B. Fibrosis in peripheral lymphatics of B16/F10 tumor implanted under the ear skin.

A, B. Two examples of intralymphatic fibrosis identified at the edge of the lymphatic tumor. B16/F10 tumor cells were implanted for 7 days under the dorsal dermis of a mouse ear. Arrows point to clusters of collagen I and collagen IV-positive matrix inclusions inside lymphatic collecting vessels. Arrowheads point to disorganized lymphatic endothelial cells with absent cell-cell junctions. The intensity of collagen IV was normalized to the signal level of weakest vascular structures present in the image. The intensity of collagen I was normalized to the average signal level of interstitial collagen. Scale bar: 50µm.

Supplementary Fig. 1A. Lymph clots are forming within lymphatic collectors in peripheral lymphatics of B16/F10 tumor implanted under the ear skin.

Three weeks after implantation of tumor melanoma cells tumor mass formed palpable solid mass under the dorsal dermis of a mouse ear. The mouse was i.v. injected with FITC-labelled fibrinogen and six hours later blood circulation was flushed with Ringer's solution to remove blood from functional vessels. After that, the circulation was fixed with zinc-fixative, and the whole skin and tumor tissue were stained for basement membrane marker perlecan. Lymphatic collector vessels were identified by the presence of characteristic valves along which vessels change their diameter, from the narrow at the afferent tubular to wide at the efferent sinusoidal sides. Peritumoral lymphatic collectors had incomplete basement membrane and were also filled by autofluorescent erythrocytes that were also present outside vessels in the tumor interstitium (top two rows). However, in contrast to non-perfused (non-functional) blood vessels (bottom row), erythrocyte presence did not overlap with clotted labeled fibrin. Scale bar: 25µm.
Supplementary Fig. 2 Comparison between active and partially active thrombin administration on intralymphatic and vascular blood clotting.

Confocal microscopy of whole-mount ear. A. Site of Intradermal injection: Injection of concentrated active thrombin (thrombin ac) results in an instant but transient blood coagulation within blood vessels around the site of injection. Except for rare events, intralymphatic clotting was restricted only to the site around the injection area, and remote collectors were never occluded. Bl - blood vessels, L - lymphatics. B. Draining lymphatics: In contrast to active thrombin, two sequential injections of the mix of partially inactivated thrombin (1:6 active and inactive ratio, thrombin in/ac) had no effect on blood vessel coagulation and induced intralymphatic clotting only in remote collectors with no effect on blood coagulation at the site of injection. Intralymphatic clotting was often associated with lymphatic leakiness around the clot location (interstitial fibrin deposits in the bottom, middle image). Scale bar: 50µm.

Supplementary Figure 3. Lymphatic regeneration by intralymphatic sprouting

Lymphatics regenerate by repopulation of ghost vessels. Eight days after photodynamic therapy (PDT), the state of lymphatic regeneration was observed by confocal microscopy on the whole mounted dermis. Ghost vessels, the remaining collector basement membrane sleeves stained with collagen IV, were filled with densely packed antigen presenting cells (CD45). Lymphatic endothelial cells (CD31, arrowheads) grow in close contact with the CD45+ cells. Scale bar: 50µm.
Supplementary Figure 4. Dendritic cells populate regenerating ‘ghost vessels’ after lymphatic-specific photodynamic therapy
A. Epidermal Langerhans cells, classic antigen presenting cells in the ear. In the epidermis, Langerhans cells create a dense, 2-dimensional and dendrite interconnected network that stains strongly for MHCII and CD11c. Basement membrane (BM) is stained with collagen IV. B. In the control region of the ear treated with photodynamic therapy (PDT), lymphatics are not populated with antigen presenting cells. C. In contrast, lymphatic collectors decellularized with PDT are densely populated with mostly double-positive MHCII+ and CD11c+ antigen presenting cells even 10 days after the treatment. Hf-hair follicles. Scale bar: 50µm.

Supplementary Figure 5. Lymphatic occlusion alone does not lead to persistent edema
Photodynamic therapy (PDT)-induced edema is transient and is resolved before lymphatic regenerate. 2 µg of Visudyne® (6x more than used to occlude the ear lymphatics) was injected in two sides of the tail with FITC-dextran. Draining lymphatics at a distance of 4 cm of the tail were irradiated with 25 J/cm² and they were imaged with IVIS every day for 15 days. A. Example tail before edema development (day 0), during its peak (day 1 to 3 after PDT) and after the edema resolution (day 5, 7). B. Graph representing the edema development as measured by tail volume, and resolution after PDT.