Targeting β1-integrin inhibits vascular leakage in endotoxemia

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Loss of endothelial integrity promotes capillary leakage in numerous diseases, including sepsis, but there are no effective therapies for preserving endothelial barrier function. Angiopoietin-2 (ANGPT2) is a context-dependent regulator of vascular leakage that signals via both endothelial TEK receptor tyrosine kinase (TIE2) and integrins. Here, we show that antibodies against β1-integrin decrease LPS-induced vascular leakage in murine endotoxemia, as either a preventative or an intervention therapy. β1-integrin inhibiting antibodies bound to the vascular endothelium in vivo improved the integrity of endothelial cell–cell junctions and protected mice from endotoxemia-associated cardiac failure, without affecting endothelial inflammation, serum proinflammatory cytokine levels, or TIE receptor signaling. Moreover, conditional deletion of a single allele of endothelial β1-integrin protected mice from LPS-induced vascular leakage. In endothelial monolayers, the inflammatory agents thrombin, lipopolysaccharide (LPS), and IL-1β decreased junctional vascular endothelial (VE)-cadherin and induced actin stress fibers via β1- and α5-integrins and ANGPT2. Additionally, β1-integrin inhibiting antibodies prevented inflammation-induced endothelial cell contractility and monolayer permeability. Mechanistically, the inflammatory agents stimulated ANGPT2-dependent translocation of α5β1-integrin into tensin-1–positive fibrillar adhesions, which destabilized the endothelial monolayer. Thus, β1-integrin promotes endothelial barrier disruption during inflammation, and targeting β1-integrin signaling could serve as a novel means of blocking pathological vascular leak.

β1-integrin | ANGPT2 | TIE2 | permeability | sepsis

Vascular stability is crucial for normal tissue homeostasis whereas decreased vascular integrity is a hallmark of serious pathological conditions, such as sepsis, acute respiratory distress syndrome (ARDS), fatal dengue fever, and malaria. Capillary leak syndromes are also a common toxicity of certain immunotherapies. A burst in the generation of proinflammatory agents, as a part of an overwhelmed host response, contributes to endothelial barrier breakdown in these diseases, promoting shock and multiorgan failure in patients (1, 2). The underlying molecular mechanisms behind pathological capillary leakage remain incompletely understood, hindering the development of targeted therapies aimed at vascular stabilization (3).

Endothelial barrier function is maintained via a dynamic coordination of endothelial cell (EC)-cell junctions, the contractile actin cytoskeleton, and EC adhesion to the underlying basement membrane in response to extracellular stimuli. Proinflammatory cytokines, microbial components such as lipopolysaccharide (LPS), thrombin, and vascular endothelial growth factor (VEGF) induce endothelial permeability by binding to their cognate receptors on ECs. These inflammatory agents activate signals that converge on common downstream pathways, including dismantling of vascular endothelial (VE)-cadherin (Cadherin 5, CDH5) from EC–EC junctions (4, 5). Small GTPases of the Rho family and myosin light chain (MLC) kinase (MLCK) also stimulate remodeling of the cortical actin cytoskeleton of stable endothelial monolayers into contractile actin stress fibers, generating cellular tension after inflammatory stimulation (4). Furthermore, the EC-derived growth factor angiopoietin-2 (ANG2, ANGPT2), which is up-regulated in diseases associated with inflammation and vascular leakage, synergizes with multiple inflammatory agents, promoting endothelial permeability (6, 7).

Integrins, transmembrane receptors that exist as heterodimers of α- and β-subunits, couple ECs to the underlying extracellular matrix (ECM) through their ectodomains while their intracellular domains engage the actin cytoskeleton by recruiting actin-binding proteins. In focal adhesions, integrins connect to the actin cytoskeleton and cellular signaling pathways through linker and adapter proteins, such as paxillin, talin, vinculin, and α-actinin (8). Collectively, integrin-mediated EC adhesion to the vascular basement membrane is known to mediate vascular stability and angiogenesis (9, 10), but less is known about the regulation of integrin-coupled EC adhesions during vascular leakage in mature vessels.

The α5β1-integrin heterodimer is a major endothelial fibronectin receptor. The α5- and β1-integrins and fibronectin are essential for vascular development (10–13). During embryonic development, β1-integrin regulates vascular sprouting and vessel lumen formation (11, 12). β1-integrin continues to play a critical

Significance

Compromised vascular integrity is associated with capillary leakage in sepsis, but effective therapies stabilizing the vasculature are lacking. Here, we show that targeting β1-integrin in vivo with inhibitory antibodies or deletion of a single allele of endothelial β1-integrin inhibits lipopolysaccharide (LPS)-induced vascular leakage in murine endotoxemia. The inflammatory agents IL-1β, thrombin, and LPS induced changes in endothelial cell–extracellular matrix (ECM) adhesion via β1-integrin, angiopoietin-2, and the adapter protein tensin-1, leading to increased endothelial cell contractility and permeability. These results indicate that β1-integrin actively promotes vascular leakage and that targeting β1-integrin signaling could be a novel means of achieving vascular stabilization in pathological vascular leak.

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role in vessel integrity during postnatal life, via stabilization of VE-cadherin in the developing EC–EC junctions of newly formed vessels (10). However, the function of β1-integrin in the mature vasculature and during vascular leakage has received less attention. Interestingly, α5β1-integrin signaling has been recently linked to chronic inflammation as it was shown to mediate harmful endothelial remodeling in a mouse atherosclerosis model (14). Furthermore, ANGPT2 was recently reported to activate α5β1-integrin, leading to endothelial monolayer destabilization (15). However, it is not known if β1-integrin–dependent EC-basement membrane adhesions contribute to acute endothelial permeability responses in vivo.

Here, we demonstrate that several inflammatory agents, including LPS, interleukin (IL)-1β, and thrombin, disrupt the endothelial barrier function via β1-integrin. Mechanistically, inflammatory agents stimulated, in an ANGPT2-dependent manner, the formation of β1-integrin– and tensin-1–positive fibrillar adhesions that destabilized endothelial monolayer integrity. Inhibitory antibodies against β1-integrin decreased inflammation-induced endothelial contractility and monolayer permeability. In LPS-induced endotoxemia in mice, β1-integrin antibodies decreased vascular leakage, protected from cardiac failure, and stabilized vascular integrity, without affecting the up-regulation of inflammatory markers or TEK receptor tyrosine kinase (TIE2) signaling. Further supporting a role for endothelial β1-integrin in vascular leakage, conditional heterozygous deletion of endothelial β1-integrin protected adult mice from LPS-induced vascular leakage. Thus, β1-integrin signaling promotes vascular leakage during inflammation and may be a potential therapeutic target for vascular stabilization in disease.

Results

Inflammatory Agents Decrease Endothelial Monolayer Integrity via β1-Integrin. To investigate how EC adhesion to the ECM affects inflammation-induced monolayer permeability, primary human dermal blood microvascular ECs (BECs) and human umbilical vein ECs (HUVECs) were stimulated with the inflammatory agents thrombin, IL-1β, and LPS. β1-integrin, which is the major integrin subunit expressed by ECs (11, 12, 15), was simultaneously inhibited with β1-integrin antibody (mAb13). mAb13 binds to the βI-domain in the headpiece of β1-integrin, resulting in stabilization of the β1-integrin in an inactive, closed conformation with decreased binding to ECM proteins (16). Consistent with this, mAb13 (10 $\mu g \cdot mL^{-1}$) inhibited EC spreading on fibronectin, and even stronger inhibition was obtained when mAb13 was combined with cilengitide, a cyclic Arg-Gly-Asp (RGD) containing pentapeptide that inhibits αvβ3-, αvβ5-, and α5β1-integrins (*SI Appendix*, Fig. S1 *A* and *B*). Similarly, incubation with a high concentration of mAb13 (10 $\mu g \cdot mL^{-1}$) interfered with cell junction organization (*SI Appendix*, Fig. S1*C*) whereas a low mAb13 concentration (0.1 $\mu g \cdot mL^{-1}$) preserved EC monolayer integrity and was used in subsequent studies.

Stimulation of BECs with thrombin (0.1 $U \cdot mL^{-1}$ for 30 min) resulted in a significant decrease in VE-cadherin in EC junctions (Fig. 1*A*), in line with previous reports (17). Whereas treatment with mAb13 (0.1 $\mu g \cdot mL^{-1}$) had no effect on VE-cadherin in unstimulated control cells, it prevented the thrombin-induced loss of VE-cadherin (Fig. 1 *A* and *B*). BEC monolayer permeability was analyzed using noninvasive electrical cell impedance monitoring. Stimulation of BECs with thrombin increased monolayer permeability, in accordance with previous publications (17), but this was prevented by mAb13 (Fig. 1*C*).

A balance between the activities of MLCK and MLC phosphatase (MLCP) determines the level of MLC phosphorylation and therefore the contractile status of ECs. Thrombin induces MLC phosphorylation, leading to endothelial contractility (4). In line with this, blebbistatin, a reversible inhibitor of nonmuscle myosin II, prevented thrombin-induced VE-cadherin loss from EC junctions (Fig. 1*D*). We next performed traction force microscopy (TFM) to measure the mechanical stress exerted by the EC monolayer. Stimulation of HUVECs with thrombin (0.1 $U \cdot mL^{-1}$, for 10 or 20 min) increased the force exerted on the matrix threefold, indicating a disrupted cell monolayer (Fig. 1 *E* and *F*). Pretreatment of the cells with mAb13 before thrombin treatment significantly attenuated the thrombin-induced increase in cellular force (Fig. 1 *E* and *F*). These results indicate that mAb13 decreased thrombin-induced EC contractility, which contributes to the dissolution of VE-cadherin from EC junctions, leading to monolayer permeability.

To confirm these results, shRNA-expressing lentiviruses were used to silence β1-integrin (shβ1) in BECs. Successful β1-integrin silencing was confirmed by Western blotting and by quantitative PCR [96% mRNA silencing compared with scrambled shRNA (shScr)] (*SI Appendix*, Fig. S2). However, ∼10% of active β1-integrin remained in focal adhesions (mostly located at the cell periphery overlapping with the cortical actin cytoskeleton), even 48 h after shβ1 transduction (Fig. 2 *A* and *B*). shβ1 did not decrease VE-cadherin in confluent monolayers, but prevented the thrombin-induced loss of VE-cadherin, enabling its retention in BEC junctions, as in the untreated, shScr-transduced control cells (Fig. 2 *C* and *D* and *SI Appendix*, Fig. S3*A*). The thrombin-induced decrease in EC barrier function was also less in the shβ1-transduced cells than in the shScr-transduced control cells (Fig. 2*E*). By contrast, β3-integrin (shβ3) silencing decreased VE-cadherin in BECs, in line with previous studies (9, 15), and shβ3 did not prevent the thrombin-induced loss of VE-cadherin (Fig. 2 *C* and *D* and *SI Appendix*, Fig. S3*A*). However, silencing of ANGPT2 (shANGPT2), which is constitutively secreted by ECs, prevented the thrombin-induced loss of VE-cadherin, in line with previous studies (Fig. 2 *C* and *D* and *SI Appendix*, Fig. S3*A*) (17).

Thrombin is a fast inflammatory mediator acting via G protein-coupled protease-activated receptors whereas IL-1β and LPS stimulate slower EC responses via the IL-1 receptor and Toll-like receptor 4, respectively (1, 18). Despite acting through distinct cell surface receptors, thrombin, IL-1β, and LPS activate partially overlapping downstream signaling pathways. We thus hypothesized that β1-integrin may play a more universal role in inflammatory monolayer destabilization and investigated whether β1-integrin mediated signals downstream of IL-1β and LPS as well. Stimulation of BECs with IL-1β (10 $ng \cdot mL^{-1}$) decreased VE-cadherin in BEC junctions at 2 h (*SI Appendix*, Fig. S4); however, this did not occur in shβ1- or shANGPT2-transduced BECs (Fig. 2*F* and *SI Appendix*, Fig. S3 *B* and *C*). LPS (10 $\mu g \cdot mL^{-1}$, 2 h)-induced actin stress fibers were significantly decreased after silencing of β1-integrin in BECs (*SI Appendix*, Fig. S5). In addition, shRNA silencing of α5-integrin (shα5), which pairs with β1-integrin to form the fibronectin receptor α5β1, decreased stress fiber formation in LPS-stimulated BECs (*SI Appendix*, Fig. S5), suggesting that both α5-integrin and β1-integrin are involved in inflammation-induced monolayer destabilization. Moreover, expression of the tight junction protein zonula occludens (ZO)-1 was decreased after IL-1β and LPS stimulation in control BECs but was preserved in ANGPT2-, α5-integrin–, or β1-integrin–silenced cells (Fig. 2*G* and *SI Appendix*, Fig. S3*D*). By contrast, silencing of β3-integrin had no protective effect (Fig. 2*F* and *SI Appendix*, Figs. S3*C* and S5*A*), and actin stress fibers were prominent in β3-integrin–silenced BECs, both in the absence and presence of LPS (*SI Appendix*, Fig. S5 *B* and *C*).

To confirm our results in ECs of different origin, primary human pulmonary microvascular endothelial cells (HPMECs) were used. Stimulation with thrombin or IL-1β disrupted the cortical actin rim and VE-cadherin junctions in shScr- but not shβ1- or shANGPT2-transduced HPMECs (*SI Appendix*, Fig. S6). These results indicate that several inflammatory agents that act via distinct cell surface receptors induce EC monolayer destabilization in a β1-integrin– and ANGPT2-dependent, but not β3-integrin–dependent, manner. In addition, inflammation-induced EC permeability and contractility can be prevented by β1-integrin inhibiting antibodies, without affecting the monolayer stability in basal conditions.

β1-Integrin Antibody Reduces Vascular Leakage in LPS-Induced Endotoxemia. To investigate whether antibody targeting of β1-integrin also improved the integrity of EC–EC junctions in vivo, we evaluated vascular leakage in a murine model of endotoxemia.

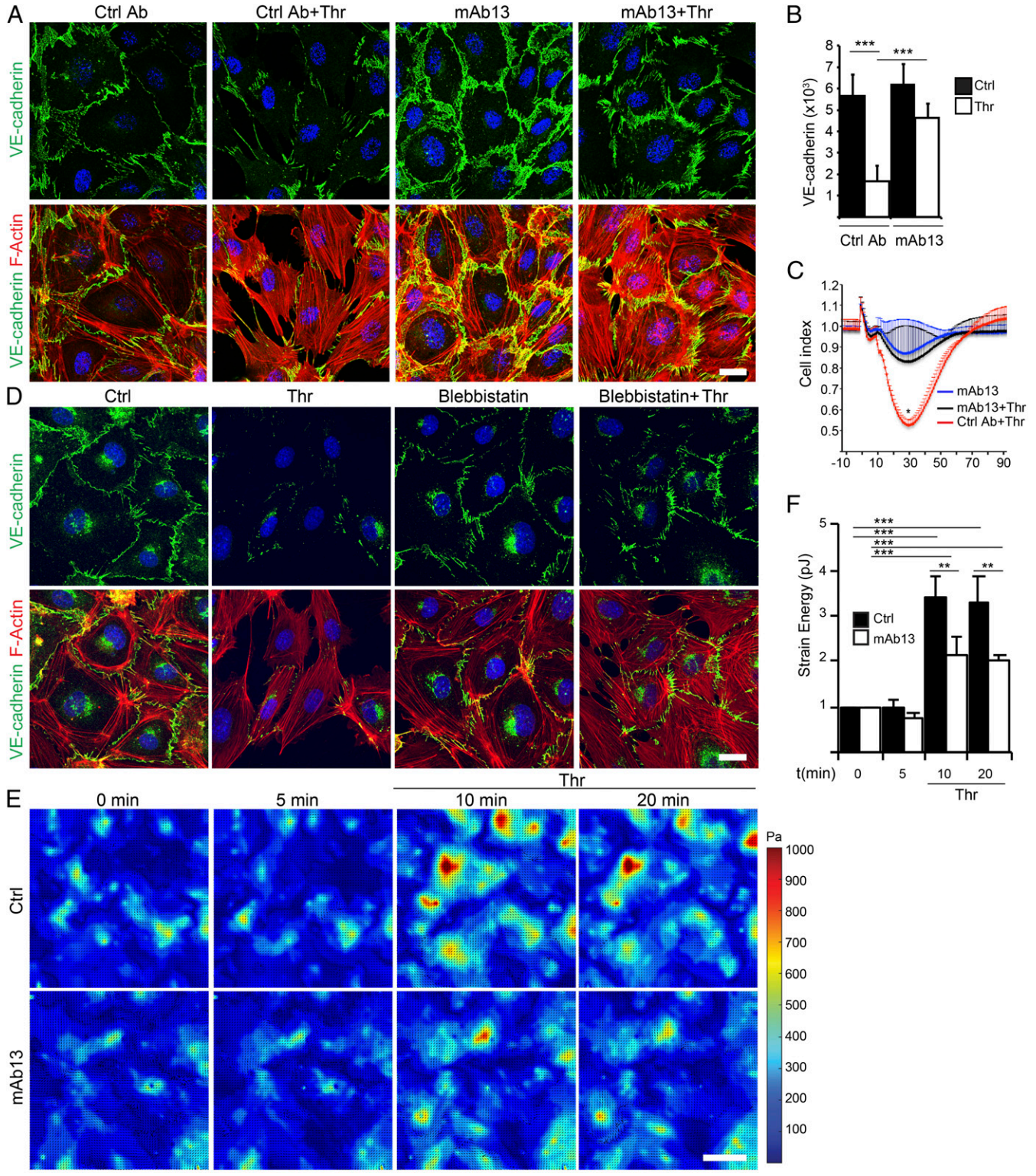

Fig. 1. β1-integrin antibody inhibits thrombin-induced endothelial monolayer destabilization. (*A*) BECs were treated with a control antibody (Ctrl Ab) or a β1-integrin inhibiting antibody (mAb13) (both 0.1 μg·mL^{-1} for 5 min), stimulated with thrombin (Thr) (0.1 U·mL^{-1} for 30 min), and fixed and stained for VE-cadherin or filamentous actin (F-Actin). (*B*) Quantification of VE-cadherin staining (150 cells per treatment. Data are the mean ± SD of $n = 3$ independent experiments). (*C*) BECs were analyzed for electrical cell impedance (represented as the cell index). Stimulation with Ab and thrombin (1 U·mL^{-1}) was initiated once the impedance reached saturating levels (set as time 0). The mean ± SD from a representative experiment is shown (four wells per treatment, $P = 0.026$). (*D*) BECs were treated with S-Blebbistatin (20 min) and then with thrombin (0.1 U·mL^{-1}, 20 min), and fixed and stained for VE-cadherin and F-actin. (*E*) Representative traction force maps exerted by HUVECs plated on fibronectin-coated polyacrylamide gels. Images were taken at baseline, at 5 min after the addition of mAb13, and after 10 and 20 min of thrombin stimulation (0.1 U·mL^{-1}). (*F*) Quantification of the mean force (strain energy, pJ). Data are the mean ± SD of $n = 3$ independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ (Tukey's test). Representative images of maximum intensity projections of confocal z-stacks obtained using a 63× objective are shown. Nuclear DAPI stain. (Scale bars: 20 μm.)

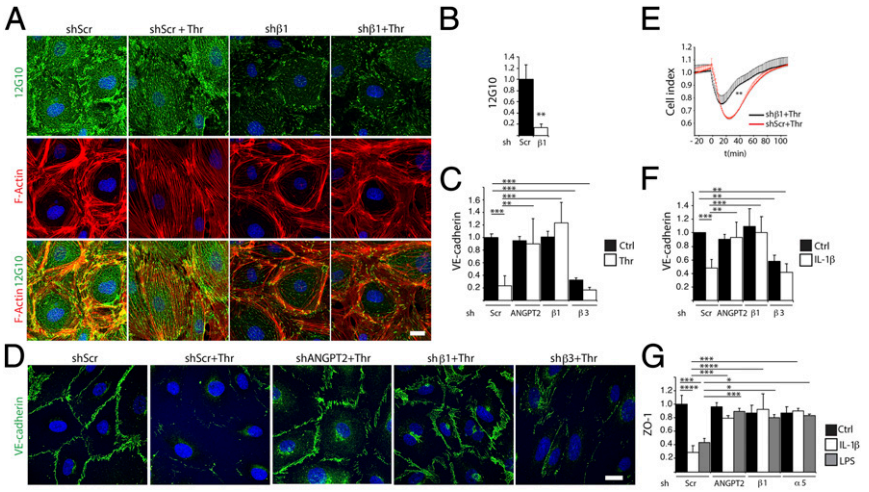

Fig. 2. Silencing of ANGPT2, β1-integrin, or α5-integrin, but not β3-integrin, inhibits thrombin, IL-1β, or LPS-induced endothelial monolayer destabilization. BECs transfected with shScr or shβ1 lentiviral vectors (*A* and *B*) were stimulated with thrombin (1 U·mL^{-1} for 30 min) and stained for active β1-integrin (12G10) and F-actin (*A*). (*B*) Quantification of 12G10 staining (350 cells per treatment, $n = 3$ independent experiments). BECs transduced with shScr, shβ1, shβ3, shANGPT2, or shα5, as indicated, were stimulated with thrombin (1 U·mL^{-1} for 30 min) (*C*–*E*), IL-1β (10 ng·mL^{-1} for 2 h) (*F* and *G*), or LPS (2.5 μg·mL^{-1} for 2 h) (*G*) and stained for VE-cadherin (*C*, *D*, and *F*) or ZO-1 (*G*). (*C* and *F*) Quantification of VE-cadherin staining (120–300 cells per treatment, $n = 3$ independent experiments). (*E*) BECs transduced with shScr or shβ1 were analyzed for electrical cell impedance (represented as the cell index). Cells were stimulated with thrombin (1 U·mL^{-1}) once the impedance reached saturating levels (set as time 0). A representative experiment is shown (four wells per treatment, mean ± SD, $P = 0.0036$). (*G*) Quantification of ZO-1–positive junction length (130 to 145 cells per treatment $n = 3$ independent experiments). Data are the mean ± SD of $n = 3$ independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ (Tukey's test). Representative images of maximum intensity projections of confocal *z*-stacks obtained using a 63× objective are shown. Nuclear DAPI stain. (Scale bars: 20 μm.)

A monoclonal antibody against mouse β1-integrin (HMβ1) (19) inhibited the spreading of murine ECs on fibronectin, similarly to mAb13-mediated inhibition of human ECs (*SI Appendix*, Fig. S7). Control antibodies or HMβ1 was injected [both at 2.5 mg·kg^{-1}, intraperitoneally (i.p.)] into mice 24 h before LPS administration, and vascular leakage was analyzed 16 h after LPS administration and 4 min after i.v. injection of fluorospheres (diameter of 100 nm, which is roughly equivalent to a protein molecular mass of 1,000 kDa). Vascular leakage was significantly increased in the tracheal and dermal vasculature in LPS-treated mice that had received control antibodies (Fig. 3 *A*–*C* and *SI Appendix*, Fig. S8). However, HMβ1 decreased the LPS-induced vascular leakage significantly without affecting the baseline vascular permeability in PBS-treated mice (Fig. 3 *A*–*C* and *SI Appendix*, Fig. S8).

To determine whether HMβ1 inhibited the permeability of lower molecular mass substances, we used 70-kDa fluorescent dextran (the approximate molecular mass of albumin). Importantly, HMβ1, but not control antibodies, significantly decreased the leakage of 70-kDa dextran in the tracheas of LPS-treated mice, indicating that HMβ1 reduced the permeability of inflamed vessels to large as well as smaller molecular mass substances (Fig. 3 *D* and *E*).

Capillary leakage can lead to multiorgan failure in sepsis, affecting organs such as the heart. LPS administration for 16 h decreased the cardiac output, as measured by echocardiography before and after LPS administration. Pretreatment with HMβ1, but not with control antibodies, significantly improved the cardiac output, as shown by the measurement of ejection fraction (EF) and fractional shortening (FS) (Fig. 3 *F* and *G*). This suggests that the decreased vascular leakage in mice receiving HMβ1 has a physiologically significant role in protecting heart function.

β1-Integrin Antibody Reduces Vascular Leakage After the Onset of Acute Inflammation. LPS induced a strong inflammatory cytokine response by increasing the circulating levels of IL-6, IL-1β, TNF-α, and ANGPT2 (Fig. 4*A* and *SI Appendix*, Fig. S9). IL-6, IL-1β, and TNF-α peaked between 1 and 3 h after LPS administration whereas ANGPT2 was increased between 3 and 16 h after LPS administration, which coincides with the decrease in *Angpt1* expression (Fig. 4*A*) (20, 21). The induction of proinflammatory cytokines in response to LPS was not decreased in mice receiving the HMβ1 antibody, indicating that HMβ1 did not diminish the LPS-induced host cytokine response (Fig. 4*A* and *SI Appendix*, Fig. S9).

Next, we tested the effect of HMβ1 on vascular leakage after the onset of systemic inflammation, which is more clinically relevant than the preventative model of HMβ1 administration. In the intervention experiment, HMβ1 or control antibodies were injected into mice 2 h after administration of LPS, when the levels of IL-6, IL-1β, and TNF-α were already increased in the circulation and in the lungs, and vascular leakage was increased (*SI Appendix*, Fig. S10). Importantly, vascular leakage was significantly decreased 16 h after LPS injection (i.e., 14 h after antibody administration) in mice treated with HMβ1, but not the control antibody (Fig. 4*B*), indicating that HMβ1 resolved the vascular leakage.

β1-Integrin Antibody Decreases Vascular Leakage Without Affecting Endothelial Inflammation. LPS administration to mice induced a threefold increase in the expression of vascular cell adhesion molecule (VCAM)-1 in the lungs at 16 h, but this was not affected by HMβ1 treatment (Fig. 4 *C*–*E* and *SI Appendix*, Fig. S11 *A* and *B*). LPS also increased neutrophil numbers in the lungs, which were also not significantly altered by the HMβ1 antibody (Fig. 4 *F* and *G* and *SI Appendix*, Fig. S11 *C* and *D*). Furthermore, a neutralizing antibody against α4-integrin, which pairs with β1-integrin to form very late antigen-4 (VLA-4), which mediates leukocyte adhesion to the inflamed endothelium via VCAM-1 (22), did not reduce LPS-induced vascular leakage (*SI Appendix*, Fig. S12).

The ANGPT-TIE signaling system regulates vascular stability during inflammation. It was recently demonstrated that TIE1 on the endothelium of blood vessels is cleaved after LPS administration and during chronic infection in mice, releasing the soluble TIE1 (sTIE1) ectodomain into the circulation. This correlated with loss of ANGPT2 agonist activity and increased vascular leakage (21, 23). Consistent with these results, sTIE1 was detected in the serum 3 h after LPS administration (*SI Appendix*, Fig. S13 *A* and *B*), and TIE1 and TIE2 protein levels were decreased by 80% and 50%, respectively, in lung lysates 16 h after LPS administration (Fig. 4 *H* and *I* and *SI Appendix*, Fig. S13*C*). However, HMβ1 did not prevent the TIE receptor loss. Furthermore, HMβ1 did not prevent the LPS-induced decrease in phospho-TIE2 (Fig. 4*J*).

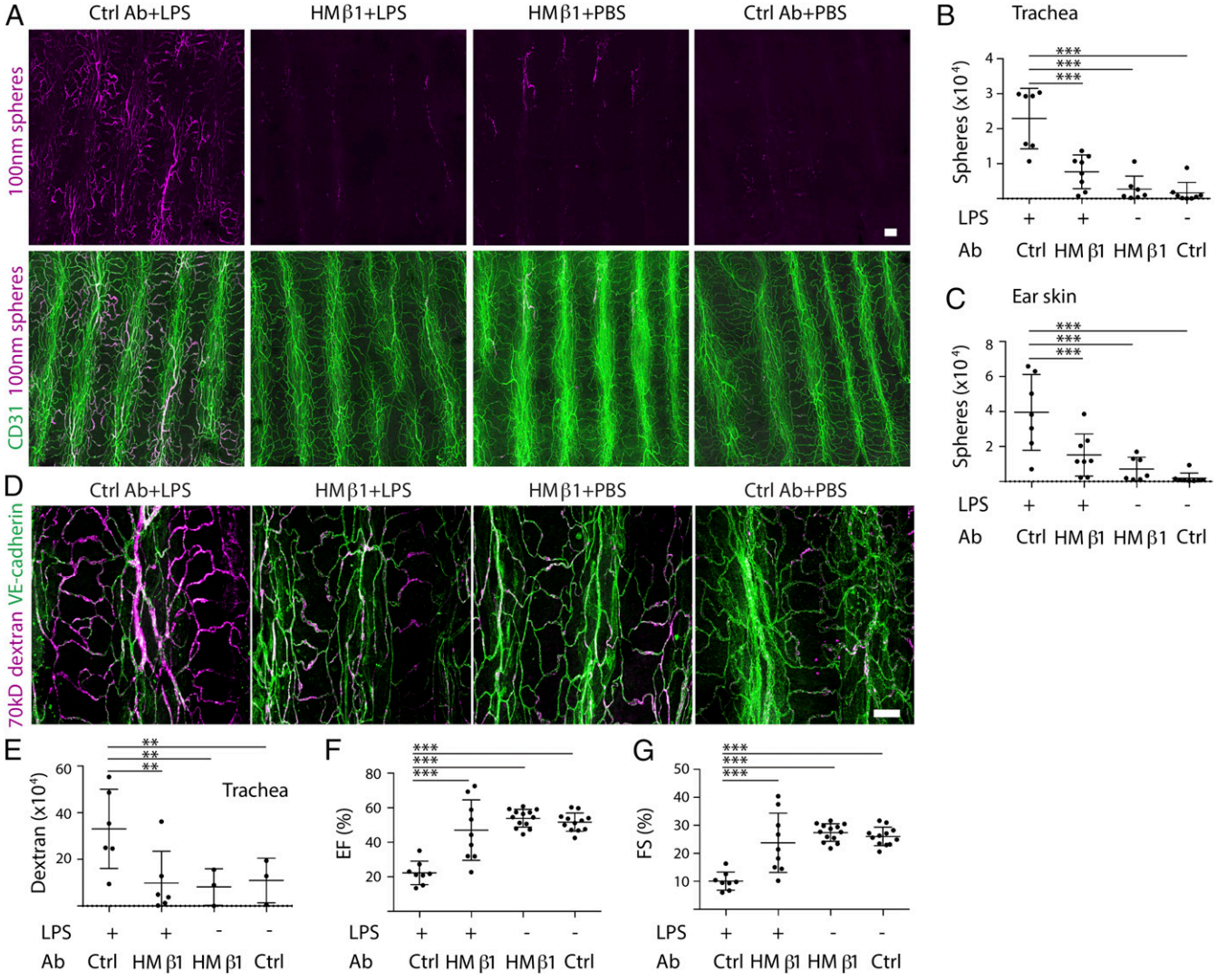

Fig. 3. β1-integrin antibody reduces vascular leakage and improves cardiac output in LPS-induced murine endotoxemia. (*A*) Analysis of leakage of 100-nm fluorospheres in mice treated with β1-integrin (HMβ1) or control antibodies (Ctrl Ab) for 24 h, followed by LPS or PBS for 16 h. Shown are representative maximum intensity projections of tile-scanned confocal z-stacks of tracheal whole mounts stained for CD31 (green), obtained using a 10× objective. Quantification of fluorosphere area in the trachea (*B*) and ear (*C*, corresponding micrographs in *SI Appendix*, Fig. S8), $n = 7$ to 8 mice per group. (*D*) Vascular leakage in mice treated as in *A* was measured using Texas red-conjugated 70-kDa Dextran. Shown are representative maximum intensity projections of confocal z-stacks of tracheal whole mounts stained for VE-cadherin (green), obtained using a 10× objective. (*E*) Quantification of fluorescent dextran area in the trachea, $n = 3$ to 6 mice per group. (*F* and *G*) Echocardiographic analysis of mice treated with HMβ1 or Ctrl Ab for 24 h. Heart function was recorded before and 16 h after administration of LPS or PBS. Ejection fraction (*F*) and fractional shortening values (*G*) are presented. $n = 8$ to 13 mice per group. Data are the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (Tukey's test). (Scale bars: 100 μm.)

Altogether, HMβ1 improved endothelial barrier function independently of the inflammation-induced adhesion molecule upregulation, neutrophil infiltration, and loss of endothelial TIE receptor signaling.

β1-Integrin Antibody Improves the Integrity of Endothelial Cell–Cell Junctions in Endotoxemia. To investigate whether the decreased vessel leakiness induced by HMβ1 after LPS administration was due to improved EC–EC junctions, endothelial junctions in the pulmonary veins, the site of the greatest amount of inflammatory cell margination, were analyzed by transmission electron microscopy (TEM). LPS treatment decreased the actin-rich, electron-dense EC junctions in the pulmonary vasculature, in comparison with PBS-treated control mice (expressed as the ratio of the electron-dense area to the total EC–EC contact length) (Fig. 5 *A–C* and *SI Appendix*, Fig. S14). However, treatment with HMβ1 significantly increased actin-rich junctions in the blood vessels of LPS-challenged mice (Fig. 5 *A–C*). Similar results were obtained when capillaries, postcapillary venules, and collecting venules in the trachea were analyzed (Fig. 5*D*). These results indicate that antibody targeting of β1-integrin in endotoxemia has a vascular stabilizing effect that is mediated via improved EC junction integrity.

Interestingly, HMβ1 was found to be localized to the vascular endothelial cell layer, overlapping with CD31 staining, but less with staining for the pericyte marker desmin, 40 h after its administration (i.e., at the end point of the analysis). The results indicate that HMβ1 homes to the vasculature for extended periods, may concentrate at sites of vascular leakage, and exerts its vascular protective effects on the endothelium (*SI Appendix*, Fig. S15). Furthermore, heterozygous *Itgb1*$^{wt/i\Delta EC}$;*Pdgfb-iCreERT2* mice, in which endothelial β1-integrin was deleted after birth or in adulthood, were protected from LPS-induced vascular leakage, indicating that endothelial β1-integrin decreases vessel integrity during inflammation (Fig. 6 and *SI Appendix*, Figs. S16 and S17).

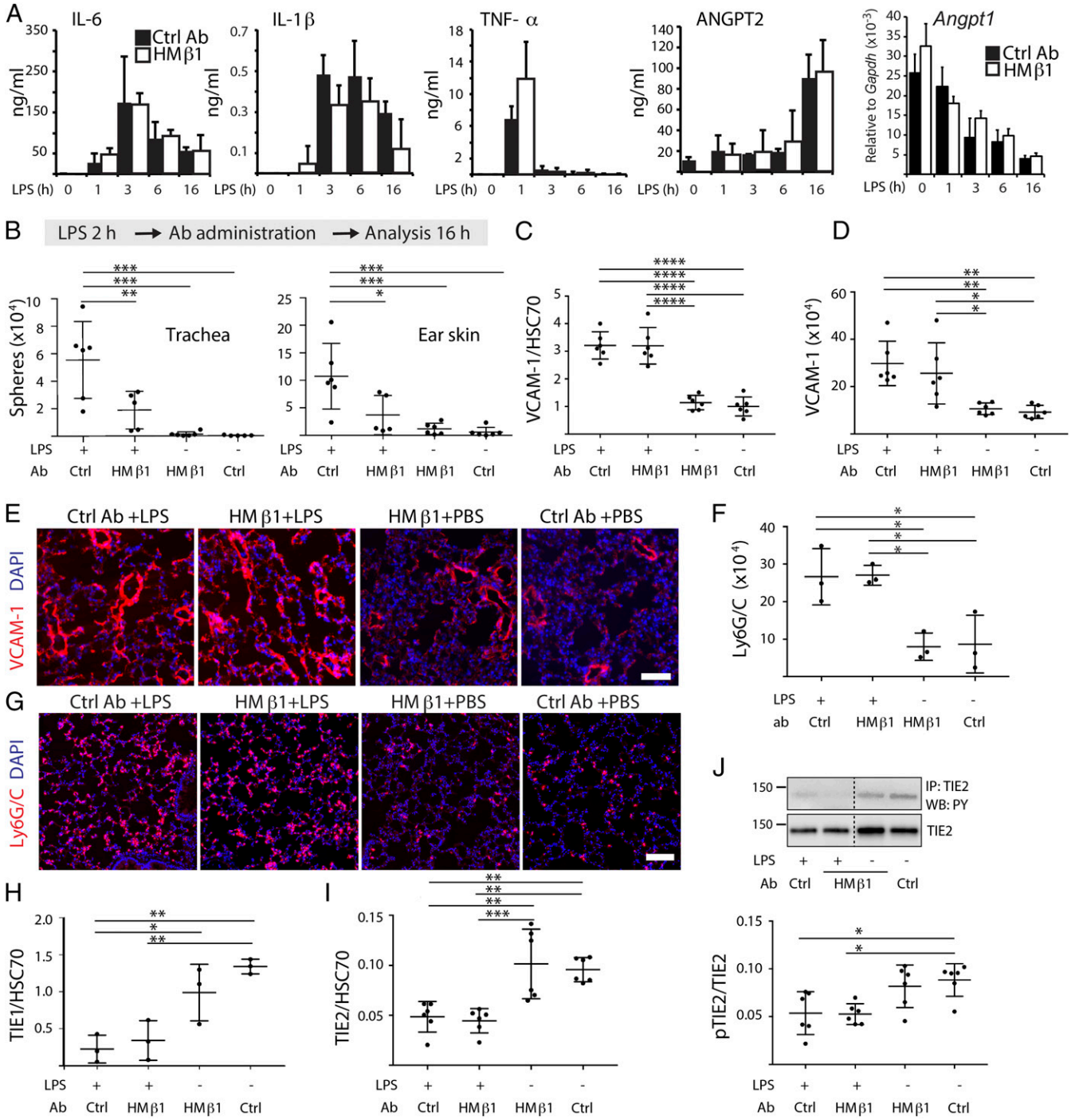

Fig. 4. β1-integrin antibody reduces vascular leakage after the onset of systemic inflammation. (*A*) Quantification of circulating levels of IL-6, IL-1β, TNF-α, and ANGPT2 in the serum and *Angpt1* mRNA in the lungs of mice challenged with HMβ1 or control antibodies for 24 h, followed by LPS or PBS for the indicated times ($n = 3$ mice per group). (*B*) HMβ1 or control antibodies were administered 2 h after the administration of LPS or PBS. Quantification of 100-nm fluorospheres in the vasculature of the trachea and ear 14 h after antibody treatment ($n = 5$ to 6 mice per group). (*C–J*) Mice were challenged with HMβ1 or control antibodies for 24 h, followed by LPS or PBS for 16 h. VCAM-1 (*D* and *E*) (n = 6 mice per group) and Ly6G/C (*F* and *G*) ($n = 3$ mice per group) staining and quantification in the lungs. Western blot analysis of VCAM-1 (*C*) ($n = 6$ mice per group), TIE1 (*H*) ($n = 3$ mice per group), TIE2 (*I*) ($n = 6$ mice per group), and phospho-TIE2 (*J*) ($n = 6$ mice per group) in mouse lung lysates. Dashed line indicates where lanes were cropped together from a single membrane. Data are the mean ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$ (Tukey's test). (Scale bars: 100 μm.)

Inflammatory Agents Promote α5β1-Integrin Translocation into Tensin-1–Positive Fibrillar Adhesions in an ANGPT2-Dependent Manner. To understand more about the mechanism through which endothelial β1-integrin induces permeability, we investigated the dynamics of EC–ECM adhesions after inflammatory stimulation of BECs. In control cells, focal adhesions dominated in the cell periphery whereas thrombin stimulation resulted in rapid formation of centrally located cell adhesions (Fig. 7 *A* and *B* and Movie S1). Similarly, upon stimulation with IL-1β (Fig. 7 *C–E* and *SI Appendix*, Fig. S18), thrombin (Fig. 7*F* and *SI Appendix*, Fig. S18), or LPS

(*SI Appendix*, Fig. S5), the active β1-integrin and the α5-integrin were enriched at centrally located, elongated matrix adhesions. The central adhesions were distinct from talin-1–positive focal adhesions that localized to the cell periphery in stable monolayers, overlapping with the cortical actin cytoskeleton (*SI Appendix*, Fig. S19*A*) (8). Instead, inflammatory agents increased staining for the scaffold protein tensin-1, whose binding site in β1-integrin overlaps with that of talin-1 (24, 25), thus marking the central adhesions as fibrillar adhesions (Fig. 7 *G* and *H*). Importantly, shRNA silencing of tensin-1 (shTNS1) prevented the IL-1β–induced loss of VE-cadherin in BECs (Fig. 7 *I* and *J* and *SI Appendix*, Fig. S19*B*). These results indicate that inflammatory agents stimulate the formation of fibrillar EC–ECM adhesions, which can be distinguished by the presence of tensin-1. In addition, tensin-1 was necessary for inflammation-induced endothelial destabilization, likely mediating the coupling of β1-integrin to actin stress fibers in fibrillar adhesions and the generation of contractile forces.

We next investigated whether the ANGPT2-TIE system was involved in the inflammation-induced formation of fibrillar ECM adhesions. Similarly to LPS-induced endotoxemia in mice, TIE1 was cleaved within 30 min after IL-1β or thrombin stimulation of ECs in culture, but this was not affected by mAb13 treatment (*SI Appendix*, Fig. S13 *D–G*). Interestingly, ANGPT2 was necessary for the LPS-induced formation of tensin-1–positive ECM adhesions (Fig. 7*K* and *SI Appendix*, Fig. S19*C*), and ANGPT2-induced VE-cadherin loss was dependent on β1-integrin expression in BECs (Fig. 7*L*). To explore the mechanism in more detail, we investigated α5β1-integrin trafficking following stimulation with LPS, IL-1β, or thrombin (*SI Appendix*, *SI Materials and Methods*) (26). LPS, IL-1β, and thrombin induced the recycling of internalized α5-integrin from intracellular vesicles into ECM adhesions; however, this did not occur in shANGPT2-transduced cells (Fig. 7*M* and *SI Appendix*, Fig. S20). In summary, inflammatory agents induced, in an ANGPT2-dependent manner, the formation of tensin-1– and α5β1-integrin–containing fibrillar adhesions that increase endothelial contractility, leading to breakdown of the endothelial barrier function (Fig. 7*N*).

Discussion

Our results identify a concept of vascular stabilization in inflammation, which is based on targeting β1-integrin to decrease vascular leakage in LPS-induced murine endotoxemia. Notably, β1-integrin inhibitory antibody was not only effective prophylactically, but also as an intervention therapy, when administered after LPS-induced vascular leakage and after the increase in serum proinflammatory cytokines (IL-6, IL-1β, and TNF-α). Moreover, β1-integrin antibody improved EC junction integrity in inflamed blood vessels, without decreasing the LPS-induced up-regulation of proinflammatory cytokines, ANGPT2, or VCAM-1. Consistent with this, β1-integrin inhibitory antibody inhibited IL-1β–induced endothelial monolayer destabilization, as well as thrombin-induced monolayer permeability and contractility.

Mechanistically, we have identified a signaling function for β1-integrin as a downstream effector of the inflammatory agents LPS, IL-1β, and thrombin in ECs. Inflammatory agent-induced loss of VE-cadherin and the formation of actin stress fibers were dependent on α5- and β1-integrins, but not β3-integrin, in ECs. Increased acto-myosin contractility has been established as an essential mechanism of endothelial barrier disruption; however, the integrins that connect the contractile actin stress fibers to the ECM during this process have not been identified. Our results indicate that β1-integrin actively promotes EC contractility in response to inflammatory agents.

We found that inflammatory agents induced the reorganization of the EC–ECM adhesions. Upon inflammatory stimulation, β1-integrin translocated into α5β1-integrin– and tensin-1–positive, centrally located, fibrillar adhesions, which were distinct from the peripheral talin-1–positive focal adhesions in stable monolayers. The maturation of focal adhesions into fibrillar adhesions has been described in fibroblasts where α5β1-integrin translocates to fibrillar adhesions to generate the necessary force for fibronectin fibrillogenesis (24, 25). Recent data further indicate that tensins maintain β1-integrin active in fibrillar adhesions, suggesting that the integrin–tensin complex supports integrin activity and mediates mechanosensitive coupling of active integrins to actin, analogously to the well-established integrin–talin–vinculin adhesions (25). The inflammatory switch to tensin-1– and α5β1-integrin–containing fibrillar adhesions described here likely mediates mechanical forces that contribute to the opening of EC–EC junctions, increasing endothelial permeability. In support of this, tensin-1 silencing inhibited the IL-1β–induced loss of VE-cadherin in EC junctions.

ANGPT2 is a context-dependent regulator of vascular stability and synergizes with inflammatory agents to induce vascular leak, via mechanisms involving inhibition of ANGPT1-TIE2 signaling

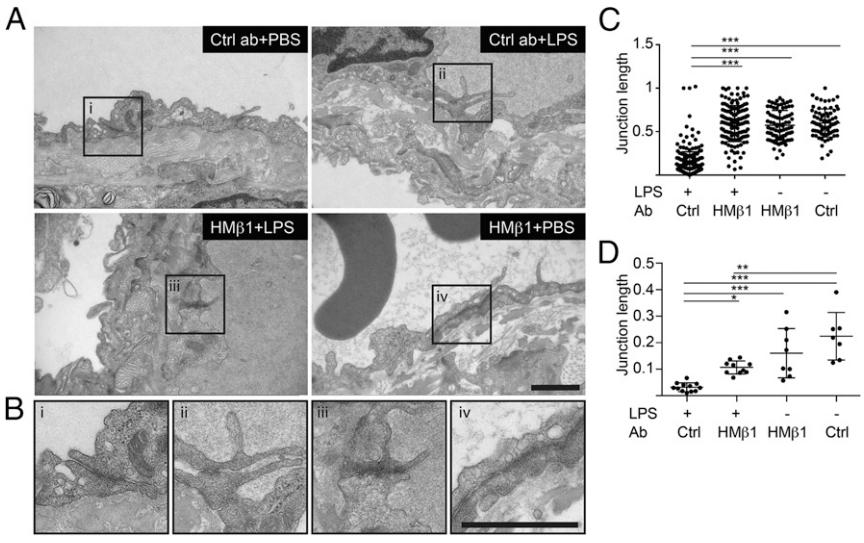

Fig. 5. β1-integrin antibody improves endothelial cell junction integrity in endotoxemia. LPS or PBS was administered to mice 24 h after the administration of HMβ1 or control antibody. (*A*) Representative transmission electron microscopic images of lungs 16 h after LPS or PBS administration. (*B*) Magnification of the area indicated in *A*. (*C* and *D*) Quantification of the electron-dense areas of endothelial cell–cell junctions relative to total endothelial cell–cell contact in the lungs (*C*) and (*D*) trachea, as described in more detail in *SI Appendix*, *SI Materials and Methods* and Fig. S14. Data are the mean ± SD of $n = 3$ mice per group. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (Tukey's test). (Scale bars: 1 μm.)

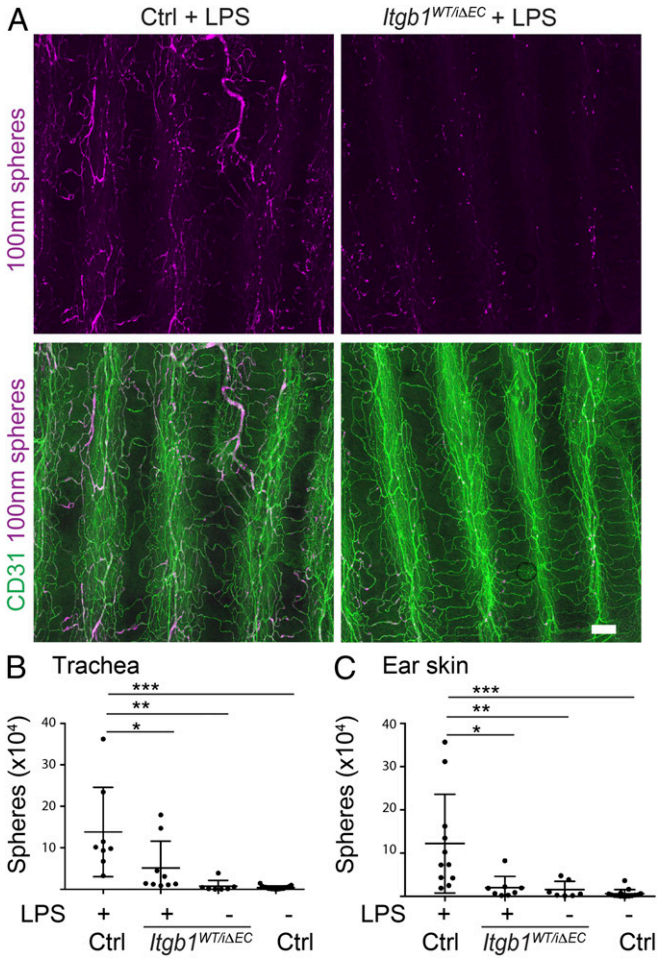

Fig. 6. Heterozygous deletion of endothelial β1-integrin protects from vascular leakage. Vascular leakage of 100-nm fluorospheres was measured in LPS- or PBS-treated Cre-positive tamoxifen-induced *Itgb1*$^{WT/fl}$(*Itgb1*$^{WT/iΔEC}$) mice or littermate controls (Ctrl) after 16 h. (*A*) Representative maximum intensity projections of tile-scanned confocal z-stacks of tracheal whole mounts stained for CD31 (green) obtained using a 10× objective. Quantification of fluorosphere area in the trachea (*B*) and ear skin (*C*). Ctrl + PBS group: $n = 15$ (6 *WT*, 5 *Itgb1*$^{WT/fl}$, 4 *Pdgfb-iCreER*^{T2} mice). Ctrl + LPS group: $n = 10$ (4 *WT*, 5 *Itgb1*$^{WT/fl}$, 1 *Pdgfb-iCreER*^{T2} mouse). *Itgb1*$^{WT/iΔEC}$ + PBS group: $n = 7$. *Itgb1*$^{WT/iΔEC}$ + LPS group: $n = 9$. Data are the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (Tukey's test). (Scale bar: 100 μm.)

and the thrombin-induced calcium response (7, 17). We previously showed that, in addition to TIE2, ANGPT2 can signal via α5β1-integrin, leading to destabilization of EC monolayers where TIE2 levels are low (15). Here, we found that ANGPT2 expression in ECs is required for LPS-, IL-1β–, and thrombin-induced monolayer destabilization, for the formation of tensin-1–positive fibrillar adhesions, and for α5β1-integrin recycling to these adhesions. Thus, our results provide evidence that ANGPT2 may mediate vascular leakage, in part via its ability to regulate EC–ECM adhesions. In line with this conclusion, transgenic overexpression of ANGPT2 in mice leads to aberrant and decreased EC-basement membrane matrix interactions (27, 28).

β1-integrin is a key regulator of blood vessel development (11, 12), and it continues to play a critical, context-dependent role in vessel sprouting and stability during postnatal life (10). However, the function of β1-integrin in the mature vasculature of adult mice has not been investigated. We found that heterozygous deletion of β1-integrin in ECs did not compromise vascular integrity but protected the mice from LPS-induced vascular leakage. In addition, short-term use of the β1-integrin inhibitory antibody did not affect EC junction integrity or induce vascular leakage in adult mice in the absence of inflammatory stimuli. This indicates that the inhibitory antibody did not affect the vascular stabilizing functions of β1-integrin under basal conditions. In agreement with the in vivo studies, we identified an effective concentration window in vitro where the β1-integrin inhibitory antibody decreased the inflammation-induced formation of actin stress fibers, loss of VE-cadherin, and EC contractility and permeability, without affecting monolayer stability under noninflammatory conditions. Consistent with these results, the transient β1-integrin silencing, which preserved 10% of the β1-integrin–positive adhesions, did not affect EC junctions of confluent monolayers in basal conditions, but protected from inflammation-induced monolayer destabilization. Thus, the present study clearly indicates that, besides the known function of endothelial β1-integrin in maintaining vessel integrity (10), β1-integrin plays a key role in actively promoting vascular leak, likely via fibrillar adhesions.

Consistent with our results in acute inflammation, others have shown that α5β1-integrin regulates endothelial activation in chronic inflammation (14). Elegant studies using knock-in mice, where the cytoplasmic tail of the fibronectin receptor α5-integrin was replaced with that of the collagen/laminin receptor α2-integrin, demonstrated that switching from α5β1 signaling to α2β1 signaling inhibited the formation of atheromas in hypercholesterolemic apolipoprotein E (*Apoe*)-deficient mice (14). Inhibiting α5β1-integrin signaling using ATN-161, a non-RGD–based peptide inhibitor of α5β1-integrin, decreased atherosclerotic plaque development in *Apoe* and low density lipoprotein (LDL) receptor (*Ldlr*) deficient mice (29, 30). The present study does not identify the possible integrin alpha subunit(s) that pair(s) with β1-integrin to promote vascular leakage; however, we show that α5-integrin was involved in mediating inflammatory responses in cultured ECs. It has been additionally reported that antibodies inhibiting αvβ5-integrin are able to decrease vascular leakage in a mouse sepsis model (31), but whether αvβ5-integrin utilizes similar mechanisms to those identified here for β1-integrin remains to be investigated.

Increased capillary leakage leading to hypovolemic shock and multiorgan failure is a key event in the pathobiology of several difficult-to-treat conditions, such as sepsis (2, 3, 6). In capillary leak syndromes that occur as a severe side effect of cancer therapies employing adoptive T cell transfer, an IL-6 receptor inhibitor has shown efficacy. A recent study demonstrated that improving the host vascular integrity in mice via administration of recombinant Slit2N, which enhances VE-cadherin localization in EC–EC junctions, increased the survival of mice during endotoxemia, polymicrobial sepsis, and H5N1 influenza (32). In another study, an ANGPT2 antibody that stimulated TIE2 activation was used to stabilize the vasculature in mouse sepsis models whereas conventional ANGPT2-TIE2 neutralizing antibodies were less effective (33). TIE2 activation was not increased following treatment with the β1-integrin antibody in our studies, indicating a TIE-independent mechanism of vascular stabilization. In our studies, β1-integrin antibody was able to prevent pathological vascular leakage in endotoxemia even when the inhibitory antibody was administered after the onset of systemic inflammation and vascular leakage. In the preventative mode, the β1-integrin antibody significantly improved the cardiac output in mice suffering from LPS-induced sepsis, suggesting that the improved vascular integrity prevented the severe drop in cardiac output. Due to the ubiquitous expression of β1-integrin in different cells and tissues and its versatile function, β1-integrin may not be a plausible drug candidate for preventing long-term vascular leakage. However, our data indicate that ANGPT2, which is increased in inflammation, can regulate EC–ECM adhesions, and therefore understanding more about this mechanism may provide a way to control β1-integrin signaling in vascular leakage in disease.

Materials and Methods

A detailed description of all materials and methods can be found in *SI Appendix, SI Materials and Methods*.

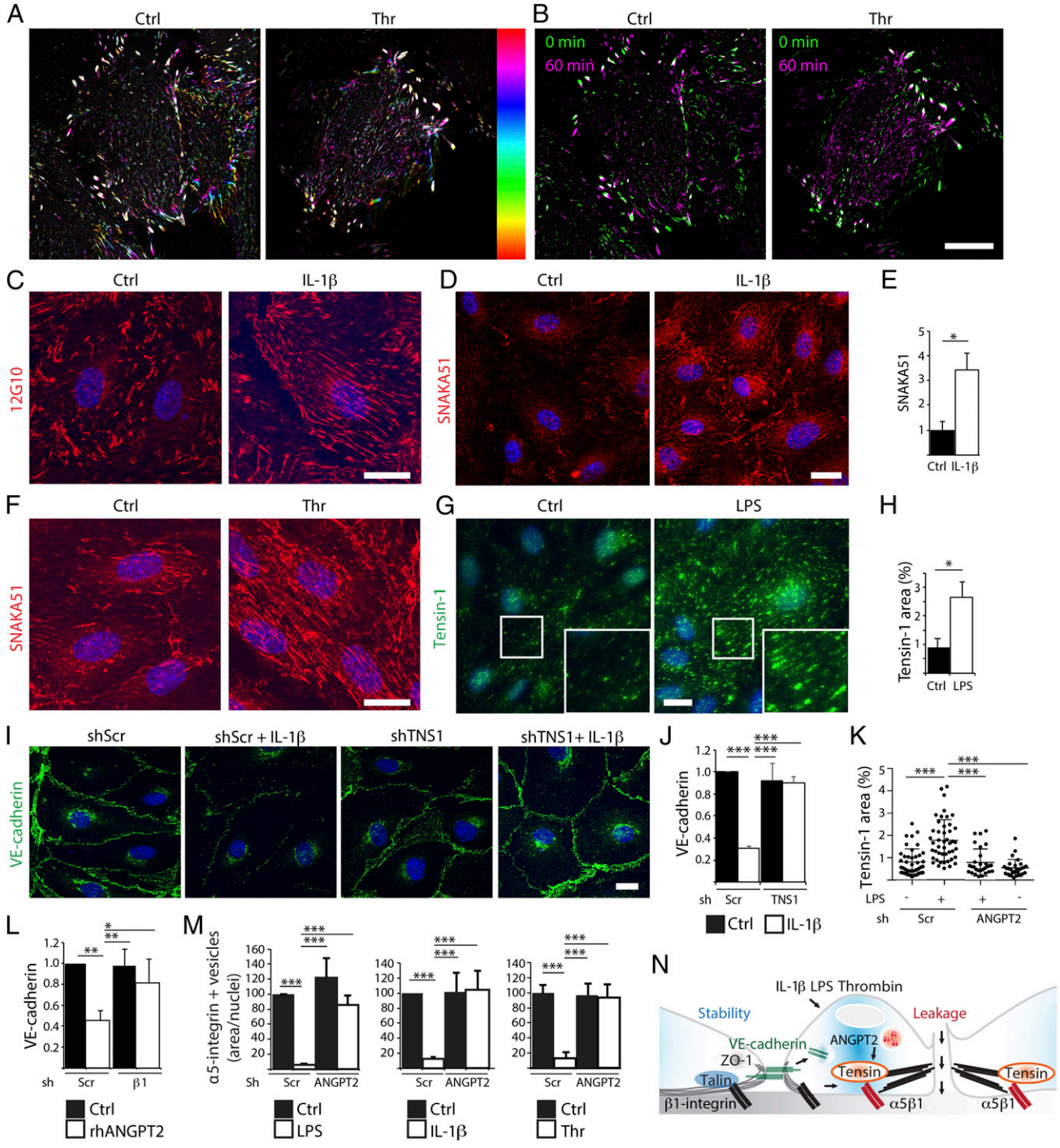

Fig. 7. Inflammatory agents stimulate the formation of α5β1-integrin– and tensin-1–positive fibrillar adhesions in an ANGPT2-dependent manner. (*A* and *B*) BECs expressing paxillin-TagGFP2 were imaged live, every 2 min for 1 h, in the presence or absence of thrombin (1 U·mL^{-1}). Representative temporal code images at times 0 to 60 min. (*B*) Merged images of time 0 (green) and 60 min (magenta). BECs were stimulated with IL-1β (2 h) (*C*–*E*) or thrombin (0.1 U·mL^{-1}, 30 min) (*F*) and stained for active β1-integrin (12G10) (*C*) or α5-integrin (SNAKA51) (*D* and *F*), or stimulated with LPS (2.5 μg·mL^{-1}, 2 h) and stained for tensin-1 (TNS1) (*G*). (*E*) Quantification of the SNAKA51-positive area (160 cells per treatment, $n = 3$ independent experiments). (*H*) Quantification of the TNS1-positive area ($n = 3$ independent experiments). (*I*) VE-cadherin staining of BECs transduced with shScr or shTNS1 and stimulated with IL-1β (2 h). (*J*) Quantification of VE-cadherin staining in *I* (220 to 250 cells per treatment, $n = 3$ independent experiments). (*K*) Quantification of the TNS1-positive area in LPS-stimulated BECs transduced with shScr or shANGPT2 (micrographs in *SI Appendix*, Fig. S19) ($n = 3$ independent experiments). (*L*) BECs transfected with shScr or shβ1 were stimulated with recombinant human (rh)ANGPT2 for 2 h and fixed and stained for VE-cadherin ($n = 3$ independent experiments). (*M*) BECs transfected with shScr or shANGPT2 were analyzed for α5-integrin recycling, as described in *SI Appendix, SI Materials and Methods*. Internalized α5-integrin was quantified in cells stimulated with LPS, IL-1β, or thrombin relative to unstimulated, shScr cells after recycling. LPS, IL-1β, and thrombin induced the recycling of internalized α5-integrin from vesicles to cell adhesions in shScr-transduced but not shANGPT2-transduced cells. $n = 3$ independent experiments. Data are the mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (Tukey's test). Nuclear DAPI stain. (Scale bars: 20 μm.) (*N*) Schematic model of ANGPT2- and α5β1-integrin–mediated endothelial destabilization in response to inflammatory stimuli. In quiescent endothelial monolayers, the cortical actin rim and VE-cadherin–positive cell junctions maintain monolayer integrity. Inflammatory agents induce the formation of α5β1-integrin– and tensin-1–positive fibrillar adhesions, in an ANGPT2-dependent manner. This stabilizes actin stress fibers, increases tension, and destabilizes endothelial cell–cell junctions via loss of junctional VE-cadherin, leading to increased permeability.

Mouse Models. All animal experiments were approved by the National Animal Experiment Board in Finland.

Analysis of Vascular Leakage in Murine Endotoxemia. Vascular leakage was analyzed as previously described (21, 23). β1- or α4-integrin antibodies, or control antibodies (all 2.5 mg·kg^{-1}), were administered to 2- to 4-mo-old male C57BL/6 mice by i.p. injection. Cre-positive tamoxifen-induced *Itgb1*$^{WT/fl}$ (*Itgb1*$^{WT/i\Delta EC}$) mice were compared with Cre-negative *Itgb1*$^{WT/WT}$, *Itgb1*$^{WT/fl}$, or *Itgb1*$^{fl/fl}$ littermates or Cre-positive *Itgb1*$^{WT/WT}$ (control mice). To induce endotoxemia, LPS (O55:B5, 11 mg·kg^{-1}) was administered i.p. for 16 h. Alexa Fluor-594–labeled fluorospheres (100 nm) or Texas red-labeled dextran (70 kDa; Invitrogen, ThermoFisher Scientific) was injected via the tail vein and allowed to circulate for 4 min, followed by sequential perfusion with PBS and 1% paraformahdehyde (PFA) in PBS via the left ventricle. Tracheas and ears were immunostained, and the fluorescence was quantified as described in *SI Appendix, SI Materials and Methods*.

Fluorescence microscopy and quantification of fluorescent and TEM images are described in *SI Appendix, SI Materials and Methods*.

Statistical Tests. For pairwise comparisons of two treatment groups, Student's *t* tests (two-tailed, unequal variance) were used, and, for comparisons of multiple groups, Tukey's multiple comparison tests were used in conjunction with ANOVA post hoc pairwise testing. A *P* value of less than 0.05 was considered statistically significant. Experimental group sizes are indicated in the figure legends.

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