Gene control of tyrosine kinase TIE2 and vascular manifestations of infections

Gene control of tyrosine kinase *TIE2* and vascular manifestations of infections

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Ligands of the endothelial-enriched tunica interna endothelial cell kinase 2 (Tie2) are markedly imbalanced in severe infections associated with vascular leakage, yet regulation of the receptor itself has been understudied in this context. Here, we show that *TIE2* gene expression may constitute a novel vascular barrier control mechanism in diverse infections. Tie2 expression declined rapidly in wide-ranging models of leak-associated infections, including anthrax, influenza, malaria, and sepsis. Forced Tie2 suppression sufficed to attenuate barrier function and sensitize endothelium to permeability mediators. Rapid reduction of pulmonary Tie2 in otherwise healthy animals attenuated downstream kinase signaling to the barrier effector vascular endothelial (VE)-cadherin and induced vascular leakage. Compared with wild-type littermates, mice possessing one allele of Tie2 suffered more severe vascular leakage and higher mortality in two different sepsis models. Common genetic variants that influence *TIE2* expression were then sought in the HapMap3 cohort. Remarkably, each of the three strongest predicted *cis*-acting SNPs in HapMap3 was also associated with the risk of acute respiratory distress syndrome (ARDS) in an intensive care unit cohort of 1,614 subjects. The haplotype associated with the highest *TIE2* expression conferred a 28% reduction in the risk of ARDS independent of other major clinical variables, including disease severity. In contrast, the most common haplotype was associated with both the lowest *TIE2* expression and 31% higher ARDS risk. Together, the results implicate common genetic variation at the *TIE2* locus as a determinant of vascular leak-related clinical outcomes from common infections, suggesting new tools to identify individuals at unusual risk for deleterious complications of infection.

Tie2 | endothelium | infection | angiopoietin | permeability

Among vascular-enriched receptor tyrosine kinases, Tie2 is unusual in at least two functional aspects. First, Tie2 phosphorylation is tightly controlled by the interplay of several proteins: a paralogous receptor, Tie1; a tyrosine phosphatase, vascular endothelial-protein tyrosine phosphatase (VE-PTP); and two secreted ligands, angiopoietin (Angpt)-1 and Angpt-2, the latter of which can act as an agonist, partial agonist, or antagonist depending upon context (1–6). Second, unlike classic growth factor receptors, Tie2 is heavily expressed and phosphorylated throughout the quiescent adult vasculature (7), suggesting that Tie2 signaling has one or more roles in vascular maintenance.

Based largely on Angpt-1 overexpression studies, Tie2 has been implicated in vascular barrier defense (8, 9). However, adult-specific deletion of Angpt-1 does not appear to trigger vascular leakage (10). Moreover, Angpt-1 has repeatedly been ascribed functions that are independent of Tie2 (11–13). Finally, observational studies in humans suffering clinical manifestations of vascular leakage have consistently shown a marked imbalance in Tie2 ligands tilting in favor of Angpt-2 (reviewed in 14). Although decreased Tie2 activity has been inferred from these reports, the role of *TIE2* gene expression has not been directly queried experimentally or in clinical settings.

This question is important not only for understanding control mechanisms of the circulatory system but also to guide the development of strategies to predict, stratify, and treat patients affected by acute vascular leakage. If tonic Tie2 signaling is indeed necessary for vascular barrier maintenance, then reducing the pool of receptors could constitute a ligand-independent means to attenuate barrier-protective signaling in the endothelium. We therefore hypothesized that the level of Tie2 expression modulates the sensitivity of blood vessels, and thereby the entire organism, to noxious stimuli. Cellular, rodent, and human genetics studies were undertaken to test this concept.

Results

Tie2 Suppression Is a Common Feature of Diverse, Leak-Associated Infectious Diseases. Experimental sepsis induces Angpt-2 expression and release, reduces Tie2 activation, and leads to vascular leakage, whereas Angpt-2 blockade or Angpt-1 administration

Significance

Major infections, such as influenza and bacterial sepsis, kill millions of individuals yearly, most commonly from complications affecting the vasculature, such as acute respiratory distress syndrome. Poor outcomes from rare infections, such as Ebola virus disease, have also been linked to the vasculature. The basis for prominent vascular involvement in infectious syndromes remains poorly understood. The present work shows that humans exhibit common, yet highly consequential, genetic variation in the ability to sustain expression of a key homeostatic vascular receptor called tunica interna endothelial cell kinase 2. The results suggest that host determinants of the molecular vascular response to infection may have a heretofore underappreciated impact on clinical outcomes. They also suggest new means to identify at-risk individuals and personalize future therapies.

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Conflict of interest statement: S.M.P. is listed as an inventor on patent applications filed by Beth Israel Deaconess Medical Center. S.M.P. has consulted for Vasomune and Eunoia. S.J.H. and K.C.K. are listed as inventors on patents filed by the University Health Network. D.J.D. is pursuing commercialization of a method to activate Tie2.

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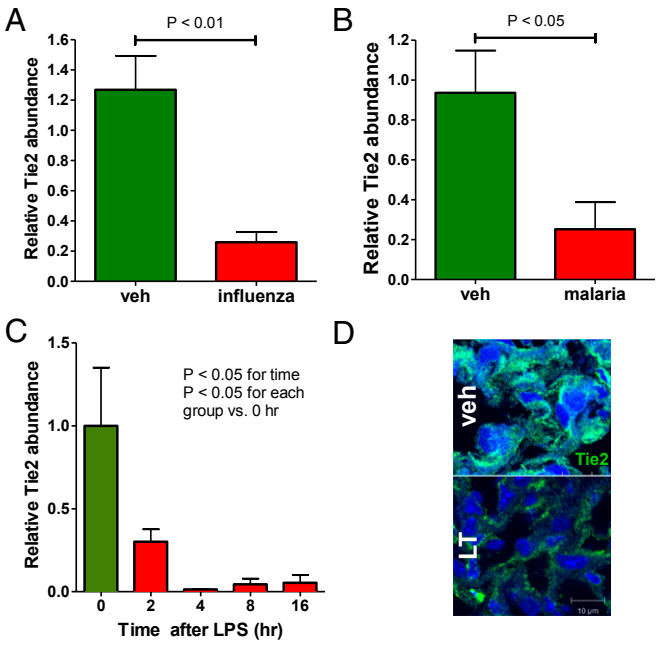

Fig. 1. Involvement of Tie2 across classes of severe infection. (*A*) Lung mRNA for Tie2 in mice 6 d after intratracheal exposure to influenza virus (n = 6–8 mice per group). (*B*) Lung mRNA for Tie2 in mice 7 d after exposure to a rodent malaria parasite, *Plasmodium berghei* ANKA Antwerpen–Kasapa (ANKA) strain (n = 7–10 mice per group). (*C*) Lung mRNA for Tie2 at indicated time points after systemic administration of Gram-negative *Escherichia coli* LPS (n = 5 mice per time point). $P < 0.05$ by ANOVA. (*D*) Lung Tie2 immunostaining 3 d after systemic administration of Gram-positive *Bacillus anthracis* LT (representative of n = 8–12 mice per group). veh, vehicle.

enhances Tie2 activation and counteracts leakage (1, 6, 15–17). The role of *TIE2* gene expression has not been fully investigated in this and related settings. We therefore studied models of viral, protozoal, and bacterial infection whose clinical manifestations derive, in part, from pronounced vascular leakage. Mice infected with influenza virus or *Plasmodium* parasites, the causative agent of malaria, exhibited, respectively, 85% and 58% decreased *TIE2* expression at the time of disease onset compared with baseline levels (Fig. 1 *A* and *B*). Tie2 transcripts also fell sharply in mouse lungs after systemic Gram-negative bacterial LPS administration (Fig. 1*C*). Finally, Tie2 was also suppressed in mouse lungs following systemic administration of the anthrax lethal toxin (LT) (18) (Fig. 1*D*). These findings show that Tie2 suppression is shared among diverse infections.

Tie2 Suppression Compromises Basal Endothelial Barrier Function. Angpt-1 has been shown to enhance barrier defense against permeability mediators present in sepsis (16, 19). However, it is debated whether this effect is dependent on signaling through Tie2 or alternative pathways (12, 13). To investigate the role of Tie2 expression in barrier defense, we transfected human microvascular endothelial cells (HMVECs) with a control lentivirus or one encoding a Tie2 shRNA and selected successive passages using puromycin. After confirming >95% cell positivity for the shRNA construct, we observed stable, dose-dependent suppression of Tie2 (Fig. S1 *A–C*). Coapplication of Angpt-1 with LPS restored barrier function in Tie2^{intact} cells but not in Tie2^{low} cells (Fig. 2*A*).

To model the flux of macromolecules that characterizes vascular leak in vivo, we applied fluorescently labeled albumin to confluent HMVEC monolayers and measured its translocation to the abluminal side (Fig. 2*B*). Tie2^{low} endothelial cells (ECs) exhibited increased albumin transit at baseline compared with Tie2^{intact} ECs (data not shown). LPS exacerbated the barrier dysfunction of Tie2^{low} ECs ($P < 0.001$ vs. Tie2^{intact} ECs). Coapplication of Angpt-1 with LPS restored barrier function when Tie2 expression was intact ($P < 0.01$ vs. LPS-treated Tie2^{intact} HMVECs). However, Angpt-1 was ineffective in Tie2^{low} ECs. Finally, because hyperpermeability due to acute inflammation arises in vivo from the development of paracellular gaps between adjacent ECs (20), we tested the effect of Tie2 suppression on such gaps. Tie2^{low} monolayers were unable to prevent the formation of paracellular gaps, and they could not respond to Angpt-1 application by closing those gaps (Fig. 2 *C* and *D*). These results show that intact Tie2 expression is necessary for basal barrier function, whereas Tie2 suppression is sufficient to weaken barrier function. Second, intact Tie2 expression is necessary for barrier defense against permeability mediators. Third, intact Tie2 expression is critical to achieve maximum barrier defense conferred by Angpt-1.

Acute Suppression of Tie2 Is Sufficient to Cause Vascular Leakage. To determine the consequences of rapid Tie2 suppression in vivo, we developed siRNA targeting Tie2 (siTie2) with backbone modifications and liposomal packaging suitable for targeted delivery to lung following i.v. injection into otherwise healthy adult animals (Fig. 3 *A–C* and Fig. S2 *A–D*). There was no dropout of endothelium, as evidenced by intact expression of the marker CD31 despite a reduction in the activated form of Tie2 (Fig. S2*E*). Instead, the barrier effector protein, vascular endothelial (VE)-cadherin, was markedly attenuated both in the microcirculation (Fig. 3 *D–I*) and in large vessels (Fig. S2*F*).Two quantitative methods, extravasation of intravascular dye and determination of relative lung water content, confirmed that Tie2 suppression in the healthy adult animal

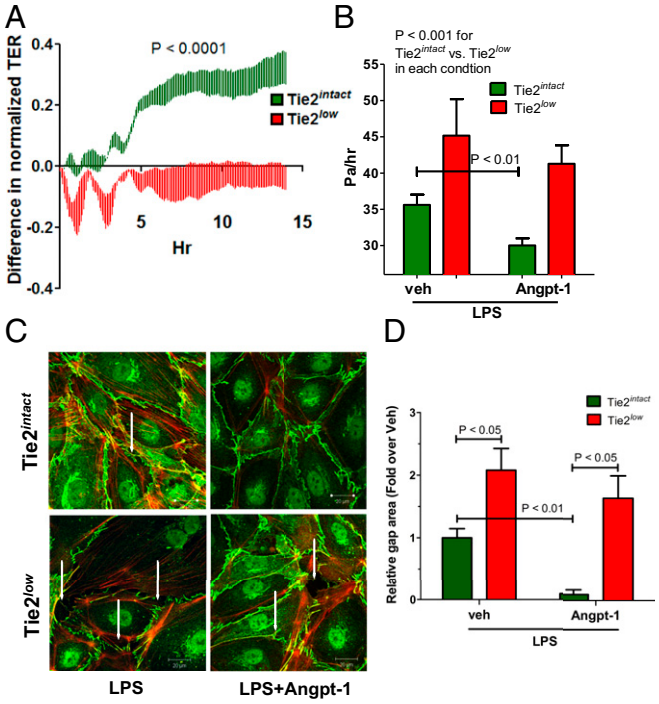

Fig. 2. Intact Tie2 expression is necessary for maximal endothelial barrier function. (*A*) Serial recordings of transendothelial electrical resistance (TER) across confluent monolayers of HMVECs treated with Angpt-1 (200 ng/mL) and LPS, and previously infected with either control shRNA lentivirus (Tie2^{intact}) or virus targeting Tie2 (Tie2^{low}) (n = 4 per group). Results were analyzed by ANOVA. (*B*) Macromolecule flux (reported as pascals per hour of FITC-labeled albumin) across confluent monolayers of Tie2^{intact} vs. Tie2^{low} HMVECs treated with LPS plus vehicle solution or Angpt-1 (200 ng/mL) (n = 4 per group). Results were analyzed by two-way ANOVA and Bonferroni posttests. (*C*) Immunostaining of Tie2^{intact} vs. Tie2^{low} HMVECs for VE-cadherin (green) and F-actin (red) 30 min after addition of LPS alone or in combination with Angpt-1 (200 ng/mL). White arrows show paracellular gaps. (*D*) Quantification of paracellular gap area for *C* as described in *SI Methods* (n = 4 independent experiments per condition).

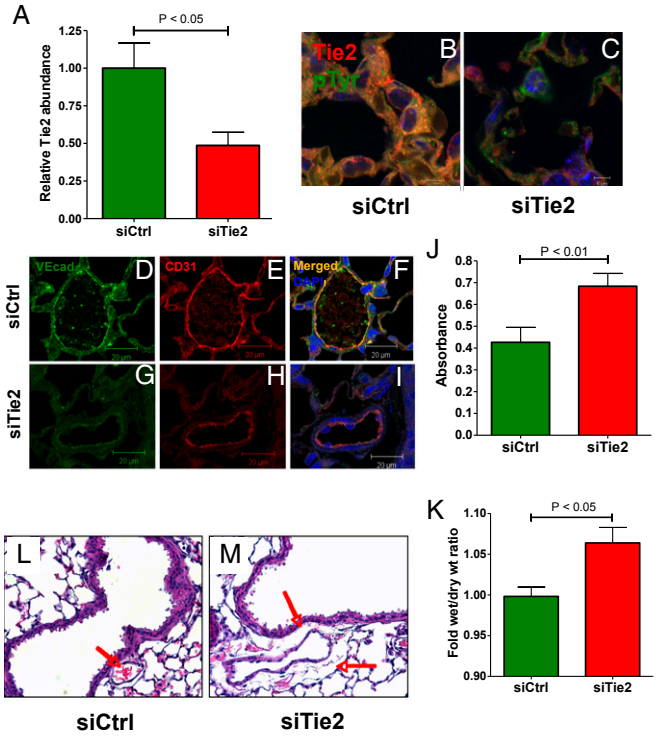

Fig. 3. Acute Tie2 suppression is sufficient to provoke leakage in vivo. (*A*) Lung mRNA following systemic injection of lipoplexed control (siCtrl) or Tie2-targeting siRNA (siTie2) (n = 4 per group). (*B* and *C*) Lung immunostaining for Tie2 (red), phosphotyrosine (green), and nuclei (blue) (representative of n = 4 per group). (*D–I*) Lung immunostaining for VE-cadherin (green), the endothelial marker CD31 (red), and nuclei (blue), representative of n = 6 per group. (*J*) Lung vascular leakage assessed by colorimetric quantification of extravasated blue dye (n = 5–6 per group). (*K*) Lung vascular leakage assessed by quantification of wet/dry weight ratios. (*L* and *M*) Lung vascular leakage assessed by development of bronchial cuffs (red arrows) filled with edema fluid, representative of n = 4 mice per group.

suffices to promote vascular leakage (Fig. 3 *J* and *K*). Furthermore, lungs of mice injected with siTie2 demonstrated widespread perivascular cuffing, a pathological sign of edema (Fig. 3 *L* and *M*).

Tie2 Suppression Mimics Infection-Associated Signaling Impairments but Does Not Induce Inflammation. Tie2 expression at the endothelial surface provides a modulatable target for affecting barrier function via VE-cadherin. To combat leakage in bacterial sepsis, activation of Akt downstream of Tie2 appears to be critical for connecting Tie2 to VE-cadherin, whereas in experimental anthrax, Tie2's barrier-protective effect appears to be signaled through the MAP kinase cascade (18). Moreover, whether Tie2 suppression contributes to endothelial inflammation has not been addressed despite data that Tie2 activation blunts inflammation (15). LPS administration promoted endothelial inflammation and Tie2 suppression (Fig. 4*A*). Activation of Akt and ERK1/2 was significantly diminished in siTie2 mice compared with control counterparts (Fig. 4*B*). This pattern of changes mimicked the effects of systemic LPS (Fig. 4*C*). Finally, siTie2 mice exhibited no induction of inflammatory adhesion molecules in vitro or in vivo (Fig. S3 *A–C*) and no infiltration of activated leukocytes (Fig. 4 *D–F*). Collectively, these results show that a fall in Tie2 expression is sufficient to promote barrier-weakening alterations in kinase signaling without inducing endothelial inflammation.

Degree of Basal Tie2 Expression Influences Susceptibility to Vascular Leakage and Mortality in Sepsis. Whereas KO mice for Tie2 are embryonically lethal, heterozygotes have ~50% reduced expression of Tie2 but no reported basal phenotype (21). Compared with septic WT littermates, septic Tie2 heterozygotes ($Tie2^{+/-}$) had lower total Tie2 protein and mRNA in the lung and less abundant phosphorylated Tie2 (Fig. 5*A* and Fig. S4 *A–D*). Tie2 heterozygotes suffered significantly worse vascular leakage in response to LPS compared with WT littermates ($Tie2^{+/+}$) (Fig. 5 *B* and *C*). Of note, we observed no genotype-dependent differences in the expression of Angpt-1, Angpt-2, or endothelial inflammatory adhesion proteins (Fig. S4 *E–I*). Finally, we compared survival of Tie2 heterozygotes with WT littermates in the LPS model and a model of ruptured appendicitis (cecal ligation and perforation). In either model, WT littermates displayed an absolute survival advantage exceeding 40% (Fig. 5 *D* and *E*). These data show that although full expression of Tie2 may be dispensable in adulthood, genetically driven changes in basal Tie2 expression influence vascular pathophysiology and survival during severe infection.

Common Shared Genetic Variants May Affect *TIE2* Gene Expression and Have an Impact on Clinical Outcomes in Severe Infection. Although vascular leakage is part of the host response to several systemic infections, only recently has the circulation been more widely appreciated as a potentially critical target for affecting clinical outcomes (22, 23). Based on the increased susceptibility of Tie2 heterozygous mice to sepsis, we hypothesized that population diversity in the set point for *TIE2* gene expression may be associated with adverse clinical outcomes. We specifically focused on the development of acute respiratory distress syndrome (ARDS), a complication characterized by excess fluid in the lung that arises, in

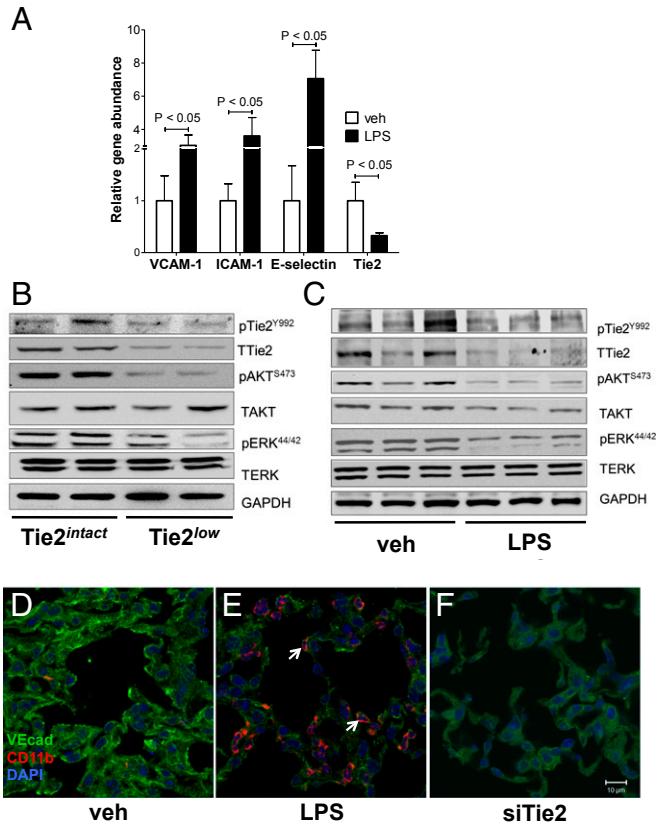

Fig. 4. Tie2 suppression recapitulates vascular signaling deficits of severe infection without affecting inflammation. (*A*) Lung mRNA 16 h after systemic LPS for markers of vascular inflammation (VCAM-1, ICAM-1, E-selectin) and for Tie2. (*B* and *C*) Western blot analysis of Akt and MAPK signaling pathways in lungs after forced Tie2 suppression and systemic LPS induction. (*D–F*) Lung immunostaining for VE-cadherin (green), a marker of activated leukocytes (CD11b, red; cells indicated by white arrows), and nuclei (blue), representative of n = 4–5 mice per group. ICAM-1, intercellular adhesion molecule 1; TAKT, total Akt; TERK, total ERK; VCAM-1, vascular cell adhesion molecule 1; VEcad, VE-cadherin.

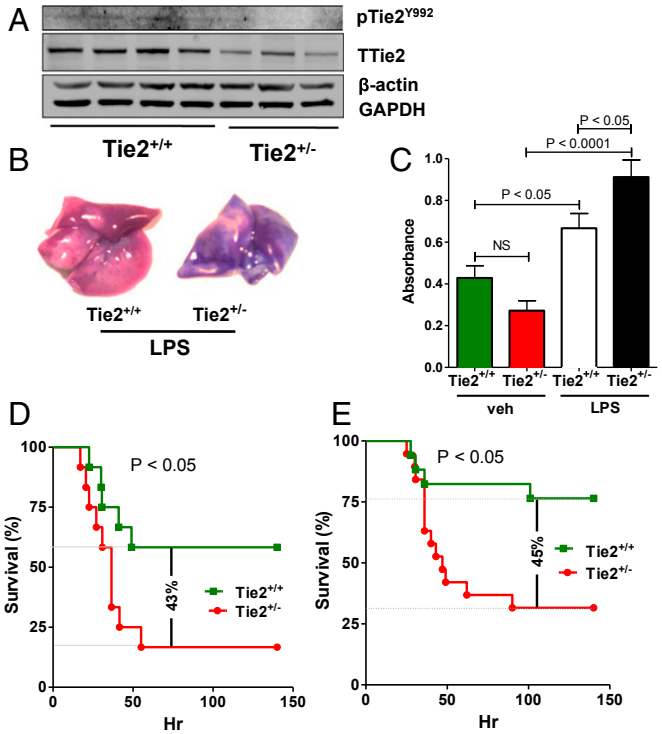

Fig. 5. Basal Tie2 expression differences exacerbate outcomes of severe infection. (*A*) Western blot analysis of activated (phosphoTie2^{Y992}) and total Tie2 in lungs of Tie2 heterozygous (Tie2$^{+/-}$) mice and WT littermates (Tie2$^{+/+}$) after systemic LPS administration. (*B*) Gross image of excised lungs after LPS and i.v. injection of blue dye bound to albumin. (*C*) Quantification of extravasated dye from indicated groups (n = 4–6 mice per group) analyzed by ANOVA with Bonferroni posttests. Kaplan–Meier analysis of survival in Tie2$^{+/-}$ vs. Tie2$^{+/+}$ mice after systemic LPS (*D*) and cecal ligation perforation (*E*).

part, from pulmonary vascular leakage. We surveyed the European population in HapMap3 for SNPs around the *TIE2* locus that could affect *TIE2* gene expression [i.e., *cis*-expression quantitative trait loci (eQTLs)] (24). In a 2-Mb interval centered on the transcriptional start site (TSS) of *TIE2*, 1,184 potential SNPs were identified (Fig. 6*A*). Of these SNPs, 16 achieved $P < 0.01$. Three of the most strongly associated SNPs, rs3780315, rs7876024, and rs581724, were located near the TSS in introns annotated as EC-specific enhancer regions, associated with heavily acetylated histones and/or EC-specific euchromatin (Fig. 6*B*). These SNPs were not in linkage disequilibrium (Fig. 6*C*). Expression of Tie2 in HapMap cell lines was significantly associated with genotype at each of the three loci (Fig. 6*C*).

We then performed focused germ-line genotype analysis at the *TIE2* locus in a large cohort of critically ill subjects (Table S1). Cases were defined by the diagnosis of ARDS according to standard definitions, whereas controls were critically ill, and thus at risk for ARDS, but they lacked this diagnosis. Each of the three SNPs from the *cis*-eQTL analysis significantly increased the risk of ARDS (Table 1). Remarkably, for each SNP, the allele associated with lower *TIE2* gene expression in HapMap3 was also the risk allele for developing ARDS. Because odds ratios were calculated after adjusting for the major clinical variables significantly associated with ARDS (i.e., age, sepsis, pneumonia), the results suggested an independent effect of these *TIE2* SNPs.

The haplotype predicted by *cis*-eQTL analysis to be associated with the highest gene expression, "*TIE2*-high" (C/G/A; haplotype 6 in Table S2), was present in 17% of the clinical cohort. In multivariable regression analysis that accounted for the strongest clinical predictors of ARDS, this haplotype was associated with 28% risk reduction ($P = 0.011$; Table S3). Indeed, compared with the haplotype predicted to be least protective, "*TIE2*-low" (A/A/G; haplotype 1 in Table S2; 42% frequency), possessing any other haplotype was associated with 31% risk reduction ($P = 0.003$; Table S4).Together, these results suggest that common, genetically determined variation in the set point for Tie2 expression may affect clinically relevant syndromes associated with vascular leakage.

Discussion

These results show that intact Tie2 expression is necessary for barrier defense, that infections associated with severe vascular leakage are characterized by a fall in Tie2 expression, and that common variations in the level of Tie2 expression may influence the human host response to severe infection. These results broaden the focus of increasingly intense research into the Angpt-Tie2 signaling axis to consider the role of gene expression control.

The human *TIE2* locus has a well-characterized promoter that consists of the 5′ flanking sequence and the first intron (25, 26). However, all three of the highly associated SNPs found in the present *cis*-eQTL analysis are located in introns 3′ to these experimentally described promoter regions. Our results do not imply that introns 9, 11, and 12 are as powerful as the 5′ flank and first intron for driving *TIE2* gene expression. Rather, they suggest that common variation in the degree to which *TIE2* is expressed relates to haplotypes (marked by polymorphisms) at other sites in the gene locus. The identified SNPs may themselves be functional or may tag novel enhancer regions in *TIE2* introns. These hypotheses remain to be determined with formal promoter regulatory studies.

The risk alleles for ARDS in an independent cohort aligned in all three instances with variants linked to reduced *TIE2* expression. Although these results require additional testing and further confirmation, they suggest that small but distinct variations in basal *TIE2* expression can interact with the environmental stress of infection to influence risk in critical illness. Whether these same polymorphisms influence outcomes, such as ventilator duration or overall survival, may require a more complex analysis that accounts for variation in major treatment variables, such as time to antibiotic initiation or frequency with which lung-protective ventilation was used (27–29). It remains to be determined whether genetic variants that lower *TIE2* expression contribute to worse survival outcomes, whereas variants that raise expression are associated with better outcomes.

The bulk of vascular leakage research in the Tie2 field has focused on the ligands. Angpt-1 protects against vascular leakage and death in experimental sepsis (15, 16). Similar protective effects arise with blockade, deletion, or depletion of Angpt-2 (1, 4, 6, 30). However, the importance of Tie2 has been diminished by the identification of integrin receptors for Angpt-1 (11–13) and, more recently, for Angpt-2 (3, 31). Integrins appear to be low-affinity, but nonetheless critical, receptors for the Angpts in several functional contexts. Polarity of ligand presentation may be important, particularly because integrins on the basolateral surface that otherwise engage basement membrane proteins would theoretically be ideally positioned to bind pericyte-derived Angpt-1 (32). An integrin-based pathway for Angpt-2–dependent endothelial destabilization may well be important in sepsis (31). The interaction of Tie2 with integrins merits further study.

Tie2-dependent barrier defense in the lung likely relies on maintenance of junctional VE-cadherin. Although transcriptional regulation of VE-cadherin has been described (33), Tie2 may also regulate localization of VE-cadherin through phosphorylation-dependent endocytosis. For example, Tie2 may limit VE-cadherin phosphorylation by sequestering the tyrosine phosphatase VE-PTP (34, 35). Alternatively, activated Tie2 has been shown to inhibit Src family kinases in the context of VEGF costimulation (36). Because activated Src also promotes VE-cadherin phosphorylation downstream of multiple proinflammatory mediators (37), intact Tie2 may be necessary to blunt Src activation. Consistent with this possibility, Tie2^{low} ECs exhibited increased activation of Src compared with Tie2^{intact} cells (Fig. S5).

Why do so many severe infections appear to share an action on Tie2? Microbe-specific pathways may alter gene expression in the endothelium, each through a unique mechanism. For example,

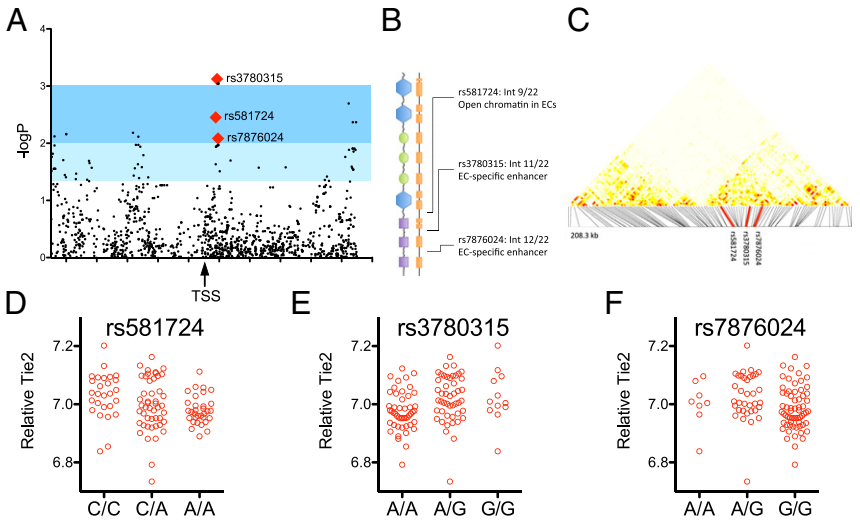

Fig. 6. Common genetic variation affects basal Tie2 expression. (*A*) Putative *cis*-eQTLs derived from HapMap 3 and transcriptomic profiling of 102 unique cell lines located within ±1 Mb of the TSS of human *TIE2* graphed as a function of genomic location (*x* axis) and *P* value (*y* axis). Shaded areas indicate regions of statistical significance. (*B*) Depiction of the extracellular domain of Tie2 (*Left*) and genomic structure (*Right*, orange bars indicate exons), with relative locations of SNPs from *A* depicted. Annotations regarding putative function are from the Ensembl Genome Browser and the UCSC Genome Browser (www.genome.ucsc.edu). (*C*) Linkage map for SNPs in the *TIE2* locus (light yellow indicates low correlation, ranging to dark orange for high correlation), with candidate SNPs indicated by red lines. (*D–F*) Normalized *TIE2* expression in cell lines from 102 subjects in HapMap 3 stratified by alleles for candidate SNPs.

anthrax LT cleaves MEK1/2 in the endothelium to affect gene expression (18), whereas Gram-negative LPSs signal through Toll-like receptor 4 to activate endothelial inflammation. There may also be secondary effects of systemic inflammation, such as reduced flow down-regulating Tie2 expression or promoting Tie2 cleavage (38, 39). Conversely, several infectious syndromes may converge on conserved aspects of the host response, such as acute inflammatory mediators. Dissecting these and intermediate possibilities will require substantial effort, but the present studies lend this question the necessary momentum for future investigation.

These results open several avenues for study. Kugathasan et al. (21) showed that compensatory changes in Angpt expression might help preserve basal Tie2 signaling when Tie2 gene expression is low. Whether similar ligand "rebalancing" is present in persons with the *TIE2* low-risk allele remains to be determined. Our results suggest that barrier maintenance is an active, rather than passive, process, requiring intact Tie2 expression. Using Tie2 expression level as a "switch" for barrier regulation constitutes a previously unidentified control mechanism that may have important implications for other vascular diseases. Activation of Tie2 by Angpt-1 suppresses inflammation in vivo (15, 16), yet reduction of basal Tie2 expression did not have an impact on cellular or molecular inflammation (Fig. 4). These findings suggest that Tie2 expression and Tie2 activation can have distinct consequences (i.e., withdrawal of basal Tie2 signaling may, alone, be insufficient to trigger inflammation). We speculate that a "second hit" in the form of a proinflammatory stimulus to microvascular barriers already weakened by reduced Tie2 expression may be needed to blossom the full proinflammatory, leaky phenotype commonly observed during severe infections. Finally, the possibility of assessing genetic susceptibility to vascular leakage opens important avenues for bedside application. For example, a *TIE2*-low haplotype suggesting leak-prone blood vessels may guide clinicians to use fluid-conservative strategies to minimize ARDS risk. Because the Tie2 pathway has been linked to an even broader array of infections than studied here, such as Ebola and Hantavirus (40, 41), the impact of genetic determinants for Tie2 protein abundance and function merits further investigation. More broadly, genetic biomarkers of vascular leak risk could catalyze drug discovery for therapies targeting the vasculature by facilitating prognostic enrichment in clinical trials.

In summary, the present findings demonstrate that Tie2 levels may be a determinant for vascular barrier function and that they implicate *TIE2* gene expression as a critical switch modulated by diverse infections associated with vascular leakage. The results suggest genetic means to identify individuals at elevated risk for infection-associated vascular complications.

Table 1. Association between SNPs in *TIE2* and ARDS risk

SNP*	Allele	$MAF^{controls}$	MAF^{cases}	MAF^{all}	OR (95% CI)†	*P* value
rs581724	C > A	0.4467	0.4932	0.4572	1.19 (1.01–1.42)	0.0414
rs3780315	A > G	0.3043	0.2575	0.2765	0.76 (0.63–0.93)	0.0060
rs7876024	G > A	0.2093	0.1662	0.1995	0.74 (0.59–0.93)	0.0091

Values >1 indicate increased odds of ARDS for the minor allele. CI, confidence interval; MAF, minor allele frequency; OR, odds ratio.
*Within 50-kb flanking region of *TIE2*.
†Estimated by logistic regression with adjustment for age, gender, sepsis, pneumonia, and blood transfusions.

Methods

Details of materials and methods are provided in *SI Methods*.

Mouse Studies. All experiments were approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center, University Health Network/Toronto General Hospital Animal Care Committee or the Harvard School of Public Health and were conducted in accordance with current institutional and national guidelines and regulations on the ethical use and care of laboratory animals in science. Tie2 heterozygous ($Tie2^{+/-}$) and WT ($Tie2^{+/+}$) littermate controls used in this study were generated as previously described on CD1 background (21).

To achieve acute Tie2 knock down in mice, lipoplexed siTie2 or control siRNA was administered via intravenous injection as described previously (42). The sense strand was 5′-auaucugggcaaaugaugg-3′, and the antisense strand was 5′-ccaucauuugcccagauau-3′, with the underlined nucleotides bearing 2′-*O*-methyl modification (42). Animals were euthanized by exsanguination under anesthesia, followed by rapid collection of organs into liquid nitrogen for further analysis.

Cell Culture. Passage 2–6 HMVECs from dermis or lung (Lonza) were cultured in EBM-2 media (Lonza) supplemented with 5% (vol/vol) FBS and growth factors according to the manufacturer's instructions. Serum starvation was performed in EBM-2 media with 1% FBS and penicillin-streptomycin for durations as noted. Unless otherwise noted for LPS experiments, cells were treated

with 100 ng/mL LPS (serotype O111:B4), CD14 (100 ng/mL), and LPS-binding protein (10 ng/mL) for the indicated period.

Genomics Methods.

Cis-eQTL analysis. To identify SNPs associated with *TIE2* expression, an online database of SNPs from HapMap3 linked to transcription expression profiles was searched in a 2-Mb interval centered on the TSS of *TIE2* (Genevar; Wellcome Trust Sanger Institute) (43). Candidate SNPs were ranked by their ANOVA-derived *P* values for association with Tie2 transcript abundance. Functional features associated with SNPs were identified in the UCSC Genome Browser and verified in the Ensembl Genome Browser.

ARDS cohort. ARDS cases and controls were identified from critically ill patients from the intensive care unit at Massachusetts General Hospital as described previously (44). The study was reviewed and approved by the Institutional Review Board of Massachusetts General Hospital. All participants or their surrogate care providers gave written informed consent. After quality evaluation, 367 ARDS cases and 1,247 at-risk controls, as well as three candidate QTL SNPs, were enrolled in the ARDS risk analysis. For focused analysis of *TIE2*, SNPs were restricted to within 50 kb of the gene locus. Statistically significant SNPs identified from the *cis*-eQTL analysis were studied further, and their effects were modeled as individual SNPs and as haplotypes with multivariable regression.

Statistical considerations for human studies. Categorical variables were described using frequency. The χ^2 test was adopted to test the difference between proportions. Multivariable regression was used to calculate odds ratios for each SNP in an additive model for ARDS risk. Covariates, including age, gender, sepsis, pneumonia, and blood transfusion, were adjusted in the statistical model. All *P* values are two-sided with a significance level of 0.05. These analyses were performed using R software version 3.2.3.

Statistical Analysis. Results are presented as mean ± SEM unless otherwise noted. A *P* value <0.05 was considered statistically significant. Means were compared by unpaired *t* test, and for groups of three of more conditions, we used ANOVA with post hoc pairwise comparisons. Analysis and graphical presentation were performed using GraphPad Prism software.

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