

# Dissociation of VE-PTP from VE-cadherin is required for leukocyte extravasation and for VEGF-induced vascular permeability in vivo

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We have recently shown that vascular endothelial protein tyrosine phosphatase (VE-PTP), an endothelial membrane protein, associates with VE-cadherin and is required for optimal VE-cadherin function and endothelial cell contact integrity. The dissociation of VE-PTP from VE-cadherin is triggered by vascular endothelial growth factor (VEGF) and by the binding of leukocytes to endothelial cells in vitro, suggesting that this dissociation is a prerequisite for the destabilization of endothelial cell contacts. Here, we show that VE-cadherin/VE-PTP dissociation also occurs in vivo in response to LPS stimulation of the lung or systemic VEGF stimulation. To show that this dissociation is indeed necessary in vivo for leukocyte extravasation and VEGF-induced vascular permeability, we generated knock-in mice expressing the fusion proteins VE-cadherin-FK 506 binding protein and VE-PTP-FRB\* under the control of the endogenous VE-cadherin promoter, thus replacing endogenous VE-cadherin. The additional domains in both fusion proteins allow the heterodimeric complex to be stabilized by a chemical compound (rapalog). We found that intravenous application of the rapalog strongly inhibited VEGF-induced (skin) and LPS-induced (lung) vascular permeability and inhibited neutrophil extravasation in the IL-1 $\beta$  inflamed cremaster and the LPS-inflamed lung. We conclude that the dissociation of VE-PTP from VE-cadherin is indeed required in vivo for the opening of endothelial cell contacts during induction of vascular permeability and leukocyte extravasation.

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Abbreviations used: ES, embryonic stem; FKBP, FK 506 binding protein; IRES, internal ribosome entry site; KI, knock-in; MLC, myosin light chain; RMCE, recombination-mediated cassette exchange; VEGF, vascular endothelial growth factor; VE-PTP, vascular endothelial protein tyrosine phosphatase.

During inflammation, leukocytes extravasating from the blood into the surrounding tissue have to overcome the endothelial barrier of the blood vessel wall. Capture and arrest of leukocytes to the luminal surface of the endothelium at sites of emigration requires the interplay of several adhesion and signaling molecules, such as selectins, chemokines, and integrin ligands (Ley et al., 2007). This is then followed by the actual transmigration step, a process called diapedesis (Vestweber, 2007; Muller, 2009).

Several adhesion molecules at endothelial cell contacts, such as platelet endothelial cell adhesion molecule 1 (Muller et al., 1993) and CD99 (Schenkel et al., 2002; Bixel et al., 2004), and some at endothelial tight junctions, such as the junctional adhesion molecules (Martin-Padura et al., 1998; Nourshargh et al., 2006) and

endothelial cell-specific adhesion molecule (Wegmann et al., 2006) are known to support leukocyte diapedesis. The fact that they are located at the interface between endothelial cells is a strong indication that they participate in the migration of leukocytes through the junctional pathway (Muller, 2001; Nourshargh et al., 2010). However, in vitro studies have shown that several of these adhesion molecules, particularly PECAM-1, junctional adhesion molecule A, and CD99 are also found surrounding leukocytes that migrate through the body of an endothelial cell on a transcellular route (Carman

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et al., 2007; Mamdouh et al., 2009), suggesting that these adhesion receptors might also participate in the transcellular diapedesis of leukocytes.

For the paracellular route, leukocytes have to overcome endothelial junctions whose opening and closure needs to be tightly controlled to facilitate extravasation and avoid leakage (Vestweber et al., 2009; Nourshargh et al., 2010). VE-cadherin is of major importance to the integrity of endothelial cell contacts (Breviario et al., 1995; Matsuyoshi et al., 1997; Crosby et al., 2005). Antibodies against VE-cadherin disrupt endothelial junctions (Corada et al., 1999), leading to an increased migration of leukocytes into the inflamed tissue (Gotsch et al., 1997). This suggests that the adhesive strength of VE-cadherin needs to be reduced during leukocyte diapedesis. We recently demonstrated that this is the case, as mice expressing a VE-cadherin- $\alpha$ -catenin fusion protein instead of VE-cadherin were resistant to the induction of vascular permeability in the skin, and leukocyte recruitment into various inflamed tissues was strongly reduced in these mice (Schulte et al., 2011).

One mechanism whereby the adhesiveness of VE-cadherin might be impaired is the tyrosine phosphorylation of the VE-cadherin-catenin complex. It was reported that the stimulation of endothelial cells with permeability-enhancing mediators such as vascular endothelial growth factor (VEGF), histamine, or thrombin triggers tyrosine phosphorylation of VE-cadherin and the associated catenins (Vestweber et al., 2009). Similarly, leukocytes adhering to endothelial cells were shown to induce tyrosine phosphorylation of the VE-cadherin-catenin complex (Allingham et al., 2007; Nottebaum et al., 2008; Turowski et al., 2008). Furthermore, mutating various tyrosines in the VE-cadherin cytoplasmic tail resulted in a decreased transmigration of leukocytes across endothelial monolayers *in vitro* (Allingham et al., 2007; Turowski et al., 2008).

We found that the endothelial-specific vascular endothelial protein tyrosine phosphatase (VE-PTP) associates with VE-cadherin and enhances its adhesive function when coexpressed in Chinese hamster ovary cells (Nawroth et al., 2002). Recently, we showed that VE-PTP is indeed required in endothelial cells for proper functioning of VE-cadherin (Nottebaum et al., 2008). A lack of VE-PTP in endothelial cells leads to increased cell layer permeability, decreased VE-cadherin adhesive function, and increased migration of leukocytes through cultured endothelial cell monolayers. Intriguingly, we showed that adhesion of leukocytes to cytokine-activated endothelial cells, and stimulation of endothelial cells with VEGF, triggers the dissociation of VE-PTP from VE-cadherin (Nottebaum et al., 2008). This correlation suggests that in situations where endothelial cell contacts have to be loosened, VE-PTP might need to dissociate from VE-cadherin to allow a modulation of the adhesive strength of VE-cadherin.

Here, we have analyzed whether dissociation of VE-PTP from VE-cadherin occurs *in vivo* and whether this process is required for the opening of endothelial junctions during leukocyte extravasation and induction of vascular permeability.

To this end, we have fused additional protein domains (FKBP and FRB\*) to the C termini of VE-cadherin and VE-PTP, which contain binding sites for a small molecule called rapalog, allowing for stabilization of VE-cadherin/VE-PTP heterodimers. DNA constructs for both fusion proteins were knocked into the VE-cadherin locus of gene-targeted mice, thereby replacing endogenous VE-cadherin. We found that LPS-triggered leukocyte recruitment into the lung, as well as *i.v.* injected VEGF, both stimulate the dissociation of VE-PTP from VE-cadherin *in vivo*. Administering rapalog into the circulation prevented this dissociation and strongly reduced VEGF- and LPS-induced vascular permeability, IL-1 $\beta$ -induced recruitment of neutrophils in the cremaster tissue, and LPS-induced infiltration of neutrophils into lungs. Thus, dissociation of VE-PTP from VE-cadherin is indeed necessary *in vivo* for the opening of endothelial cell contacts in both processes.

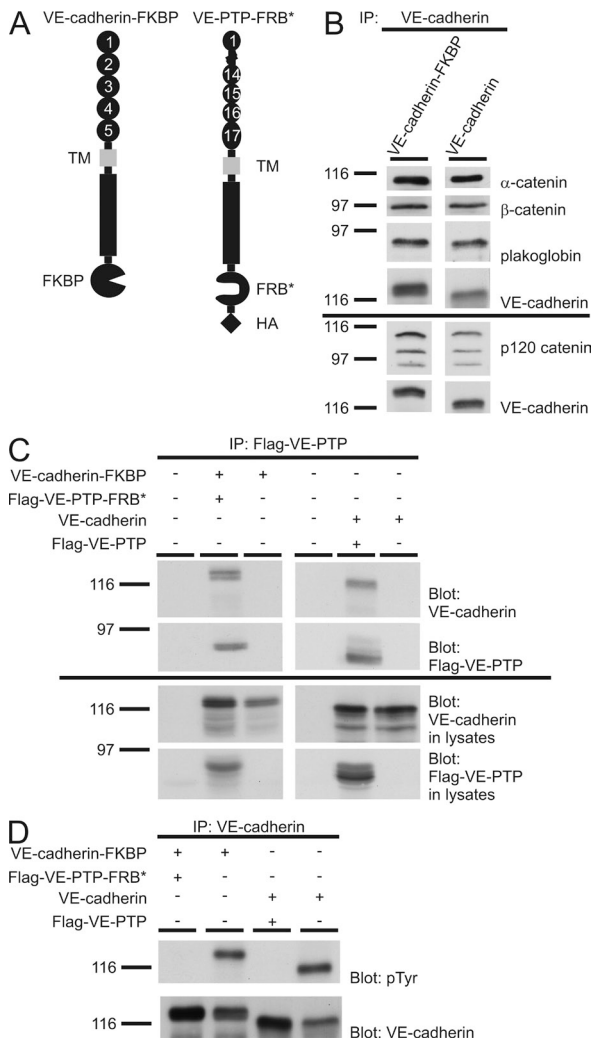
## RESULTS AND DISCUSSION

### Characterization of VE-cadherin-FKBP and VE-PTP-FRB\* fusion proteins

We have recently found that two different stimuli thought to destabilize endothelial cell contacts, the binding of leukocytes to cultured endothelial cells and the growth factor VEGF, each stimulate the dissociation of VE-PTP from VE-cadherin (Nottebaum et al., 2008). To test whether this dissociation is required for leukocyte extravasation and induction of vascular permeability, we generated modified forms of VE-cadherin and VE-PTP to prevent their dissociation. The rapamycin-binding domain of the FK 506 binding protein (FKBP) was fused to the C terminus of VE-cadherin (VE-cadherin-FKBP), and a mutated form of the rapamycin binding domain of mammalian target of rapamycin (FRB\*), containing an HA-tag at its C terminus, was fused to the C terminus of VE-PTP (VE-PTP-FRB\*; Fig. 1 A). Delivery of a nonimmunosuppressive rapamycin analogue (rapalog) that does not bind mammalian target of rapamycin should simultaneously bind to the rapamycin domains of FKBP and FRB\*, thus stabilizing the interaction between the VE-cadherin and VE-PTP fusion proteins.

To test whether the additional protein domains would affect the functions of VE-cadherin and VE-PTP, we transfected the fusion proteins into COS-7 cells. As shown in Fig. 1 B,  $\beta$ -catenin, plakoglobin,  $\alpha$ -catenin, and p120 catenin co-precipitated with VE-cadherin-FKBP in a similar manner as with wild-type VE-cadherin. In addition, VE-PTP-FRB\* and VE-cadherin-FKBP interacted to the same extent with each other as do the corresponding wild-type proteins (Fig. 1 C). This was tested with the shortened form of VE-PTP (Flag-VE-PTP) lacking the 16 N-terminal FNIII-like repeats of VE-PTP, known to interact with VE-cadherin as efficiently as full-length VE-PTP (Nawroth et al., 2002). Finally, expression of VEGF-R2 together with VE-cadherin-FKBP in COS-7 cells resulted in tyrosine phosphorylation of the fusion protein similar to WT VE-cadherin (Fig. 1 D, lanes 2 and 4). Flag-VE-PTP-FRB\* was able to

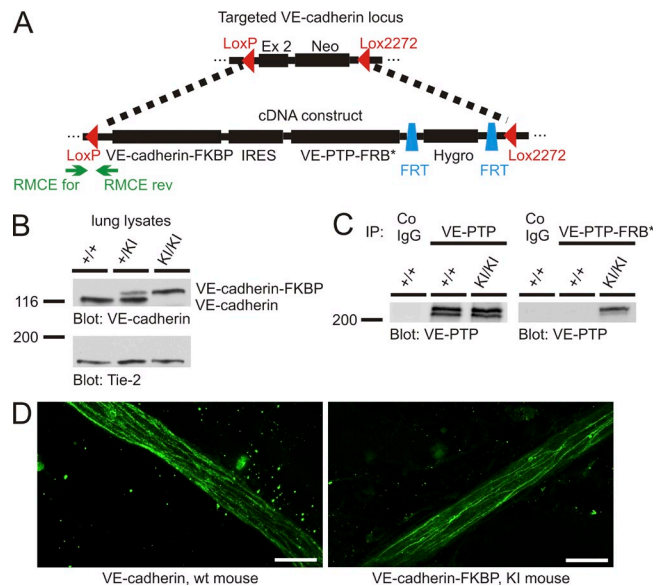
reverse this phosphorylation of VE-cadherin-FKBP with similar efficiency as WT Flag-VE-PTP dephosphorylated WT VE-cadherin (Fig. 1 D, lanes 1 and 3). Collectively, we conclude that the additional FKBP and FRB\* domains do not affect the molecular interactions of VE-cadherin and VE-PTP.



**Figure 1. VE-cadherin-FKBP and Flag-VE-PTP-FRB\* show the same characteristics as WT proteins.** (A) Schematic illustration of VE-cadherin-FKBP and VE-PTP-FRB\* transmembrane domain (TM) and HA-tag (HA). (B) VE-cadherin or VE-cadherin-FKBP were expressed in COS-7 cells. VE-cadherin was immunoprecipitated, and the indicated proteins were detected by immunoblotting. The horizontal line indicates different experiments. (C) The indicated proteins were expressed in COS-7 cells, and Flag-VE-PTP or Flag-VE-PTP-FRB\* were immunoprecipitated, followed by analysis for Flag-VE-PTP and co-precipitated VE-cadherin-FKBP or VE-cadherin by immunoblotting. Expression of transfected proteins was controlled by immunoblots of total cell lysates. (D) VEGFR-2 was coexpressed with the indicated proteins in COS-7 cells. VE-cadherin was immunoprecipitated, followed by immunoblotting for phosphotyrosine and VE-cadherin. Data are representative for two (B), six (C), and three (D) independent experiments.

**Generation of transgenic mice expressing VE-cadherin-FKBP and VE-PTP-FRB\* under the endogenous VE-cadherin promoter**

To test whether the dissociation of VE-PTP from VE-cadherin is a prerequisite for the destabilization of endothelial cell contacts *in vivo*, we generated knock-in (KI) mice expressing VE-cadherin-FKBP and full-length VE-PTP-FRB\* specifically under the endothelial specific VE-cadherin promoter. To this end, the ATG-containing exon 2 of VE-cadherin was flanked in mouse embryonic stem (ES) cells with two incompatible loxP sites (Fig. 2 A). Based on recombination-mediated cassette exchange (RMCE), these two loxP sites allowed exon 2 to be replaced by any cDNA construct that was also flanked by such a pair of incompatible loxP sites upon co-transfection with the recombinase Cre. We designed a KI



**Figure 2. Generation of transgenic mice expressing VE-cadherin-FKBP and VE-PTP-FRB\*.** (A) Schematic illustration of the RMCE approach. The gene replacement cassette contains cDNAs for VE-cadherin-FKBP and full-length VE-PTP-FRB\* separated by an IRES site, as well as a Hygromycin cassette, flanked by two FRT sites. The cassette is flanked by two incompatible loxP sites. Using RMCE, the ATG-containing exon 2 of VE-cadherin was replaced by the cDNA insertion cassette. Positions of the primers for PCR-genotyping are indicated. (B) Lung lysates of either wild-type (+/+), heterozygous (+/KI), or homozygous mutant (KI/KI) mice were immunoblotted for VE-cadherin and as loading control for Tie-2. (C) Endogenous VE-PTP was immunoprecipitated with anti-VE-PTP-C and VE-PTP-FRB\* was immunoprecipitated with anti-HA antibodies from lung lysates of homozygous KI (KI/KI) or WT (+/+) mice. Immunoprecipitations with goat IgG (CoIgG) were performed as a control. Precipitates were analyzed by immunoblotting with antibodies against the extracellular domain of VE-PTP. Note that KI mice showed only slightly increased overall amounts of VE-PTP compared with WT mice (left) because the anti-VE-PTP-C antibodies bind to VE-PTP-FRB\* only very weakly. (D) Whole mount staining of venules in cremaster from WT mice (left) and KI mice (right) for VE-cadherin or VE-cadherin-FKBP, respectively. Bars, 20 μm. Data are representative for three (B and C) and two (D) independent experiments.

construct flanked by these two loxP sites, containing the cDNAs of VE-cadherin-FKBP and VE-PTP-FRB\* separated by an internal ribosome entry site (IRES; Fig. 2 A). ES-cells were analyzed by PCR and Southern blots confirming correct homologous recombination (Fig. S1).

The resulting KI mice were viable, fertile, and healthy. As documented by immunoblotting of lung lysates, endogenous WT VE-cadherin was no longer expressed and VE-cadherin-FKBP was expressed at the same level as VE-cadherin in WT mice (Fig. 2 B). Junctional staining for VE-cadherin-FKBP in whole mounts of cremaster tissue was indistinguishable from VE-cadherin staining of WT mice (Fig. 2 D). Mobility and junctional recruitment of VE-cadherin and VE-cadherin-FKBP were similar, because the adhesion-blocking antibody BV13 against VE-cadherin removed both proteins from junctions with similar efficiency in primary isolated endothelial cells from lungs of WT and of KI mice (Fig. S2).

As the cDNA for VE-PTP-FRB\* was inserted into the VE-cadherin locus, expression of endogenous WT VE-PTP was unaffected. Expression levels of VE-PTP-FRB\* and endogenous WT VE-PTP were too low for direct analysis of lung lysates in immunoblots. The only antibody well suited for immunoprecipitation was one directed against the C terminus (anti-VE-PTP-C), which precipitated VE-PTP well but bound to VE-PTP-FRB\* only very weakly. To compare the expression levels of VE-PTP-FRB\* and endogenous VE-PTP, we precipitated VE-PTP with anti-VE-PTP-C, and VE-PTP-FRB\* with an anti-HA-tag antibody from lung lysates, followed by immunoblotting with antibodies that recognized both proteins equally well (Fig. 2 C). Taking into account the different binding efficiency of the anti-VE-PTP-C and anti-HA-tag antibodies determined in immunoprecipitations of transfected COS7 cells (unpublished data), we determined that the expression level of VE-PTP-FRB\* was 30% of the level of endogenous WT VE-PTP. Because of the large molecular weight of VE-PTP (350/265 kD doublet; Bäumer et al., 2006), the small FRB\* domain did not cause a detectable molecular weight shift by SDS-PAGE.

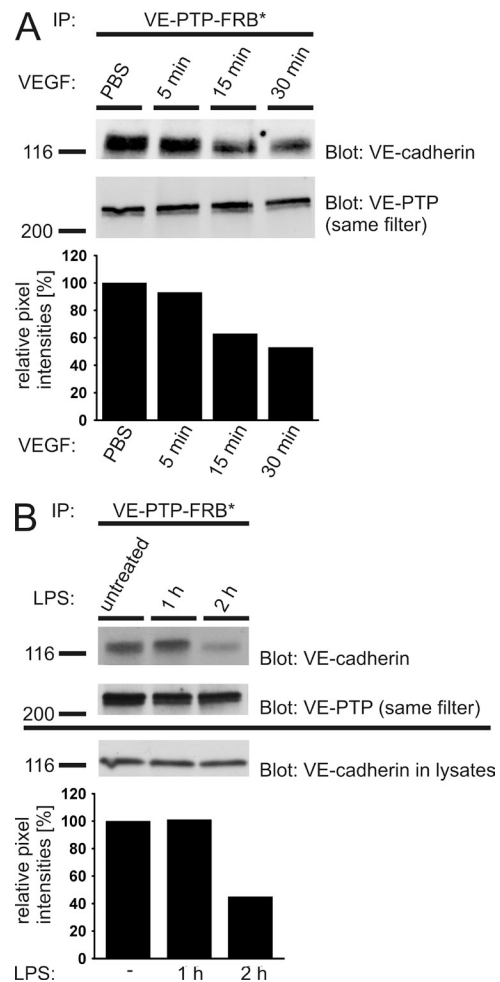
### VE-PTP dissociates from VE-cadherin in vivo after VEGF stimulation and induction of leukocyte extravasation

Because the dissociation of VE-PTP from VE-cadherin upon leukocyte docking or stimulation of endothelial cells with VEGF had only been demonstrated in vitro (Nottebaum et al., 2008), we tested whether this dissociation also occurs in vivo. KI mice were injected i.v. with 3  $\mu$ g VEGF in 100  $\mu$ l PBS or with PBS alone as a control. At 5, 15, and 30 min after the injections, VE-PTP FRB\* was immunoprecipitated from lung lysates, and precipitates were analyzed for VE-cadherin-FKBP by immunoblotting. The amounts of VE-cadherin-FKBP co-precipitated with VE-PTP FRB\* were significantly reduced at 15 and 30 min after VEGF treatment, whereas the precipitated levels of VE-PTP-FRB\* were equal (Fig. 3 A).

To test whether leukocytes would induce the dissociation of VE-PTP FRB\* from VE-cadherin-FKBP in vivo, we induced pulmonary inflammation in WT mice by exposing the

mice to nebulized LPS for 1 h. Mice were sacrificed either immediately or 1 h later, and lungs were processed as described for Fig. 3 A. Immunoblotting for VE-cadherin-FKBP revealed that its interaction with VE-PTP-FRB\* was reduced by  $\sim$ 60% 2 h after the onset of LPS stimulation. The precipitated levels of VE-PTP-FRB\* and the expression levels of VE-cadherin-FKBP in the lung lysates were unchanged (Fig. 3 B).

We conclude that VEGF stimulation and stimulation of leukocyte extravasation by LPS leads to the dissociation of VE-PTP from VE-cadherin in vivo, suggesting that this dissociation is indeed a physiological process that accompanies the opening of endothelial junctions.



**Figure 3. Dissociation of VE-PTP FRB\* from VE-cadherin-FKBP occurs in vivo after application of VEGF or LPS.** (A) Homozygous KI mice received 3  $\mu$ g VEGF i.v. and were sacrificed at the indicated time points. VE-PTP FRB\* was immunoprecipitated from lung lysates and precipitates were blotted for VE-PTP and VE-cadherin. (B) KI mice were untreated or inhaled an LPS aerosol for 1 h (3.5 mg/7 ml). LPS-treated mice were sacrificed either immediately after LPS exposure or left untreated for an additional 1 h. Lungs were analyzed at the indicated time points after starting LPS treatment. Degradation of VE-cadherin was controlled by immunoblotting total lung lysates (bottom). Data are representative of three independent experiments (A and B).

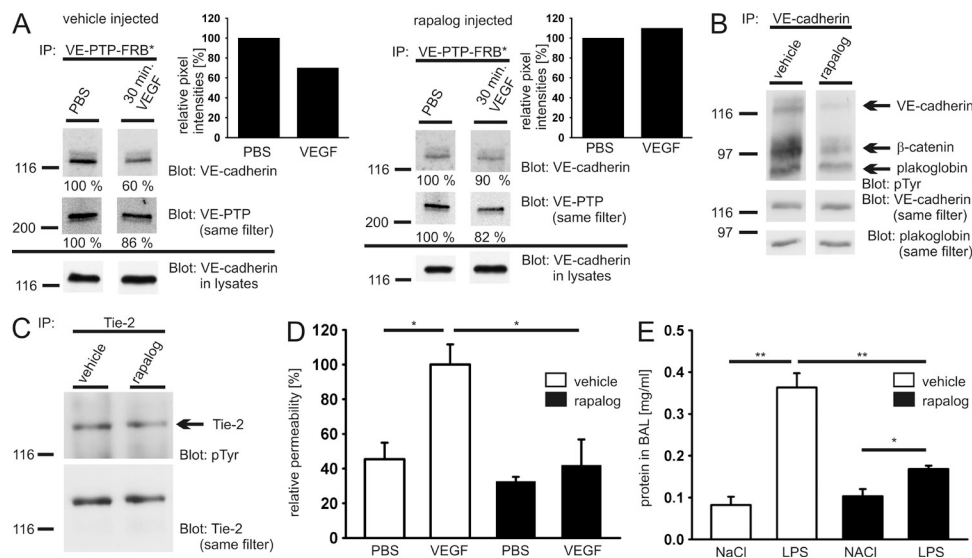
### Dissociation of VE-PTP from VE-cadherin is required for the induction of vascular permeability by VEGF

To test whether the rapalogs would indeed block the VEGF-induced dissociation of VE-cadherin-FKBP and VE-PTP-FRB\* *in vivo*, we injected KI mice *i.v.* with 250  $\mu$ g rapalog or vehicle 8 and 4 h before the assay. Subsequently, VEGF or PBS was administered *i.v.*, and 30 min later, lung lysates were immunoprecipitated for VE-PTP-FRB\*. Immunoprecipitates were first blotted for VE-cadherin-FKBP, and the same filters were subsequently reanalyzed for VE-PTP-FRB\*. In addition, lung lysate aliquots were set aside and analyzed directly by immunoblotting for VE-cadherin-FKBP, to control for degradation. As shown in Fig. 4 A, the amount of VE-cadherin-FKBP (standardized to the amount of precipitated VE-PTP-FRB\*) that was co-precipitated was reduced by 31% upon VEGF stimulation in the presence of vehicle, whereas VEGF no longer reduced the co-precipitation efficiency if the mice had been treated with the rapalog. Thus, the rapalog does inhibit VEGF-induced dissociation of VE-PTP-FRB\* from VE-cadherin-FKBP *in vivo*.

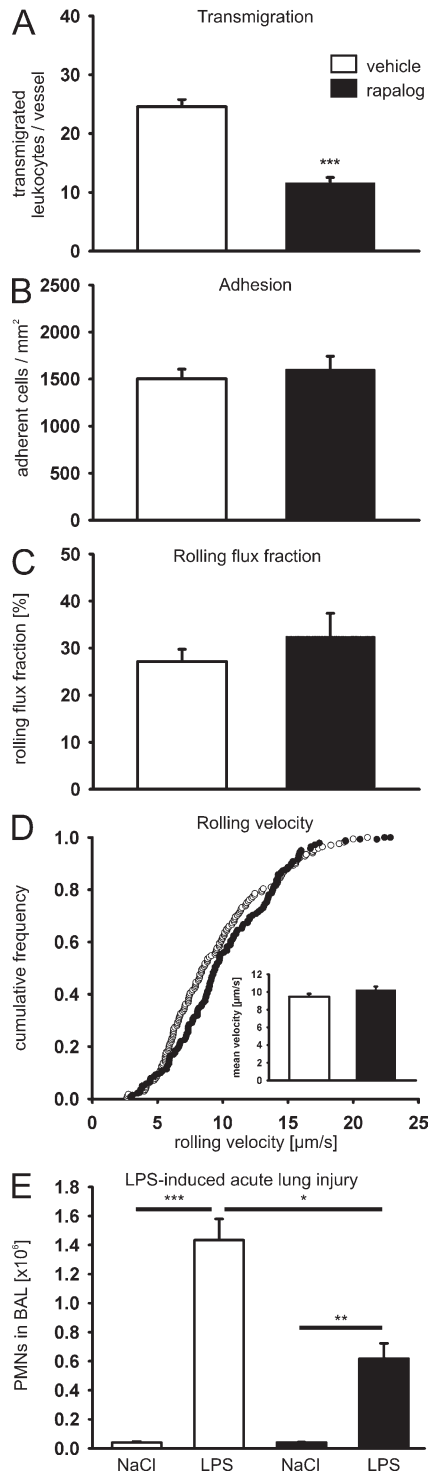
To assess whether the rapalog affects tyrosine phosphorylation of components of the VE-cadherin-catenin complex,

but not of other VE-PTP substrates such as Tie-2, we treated the animals with VEGF and rapalog or vehicle, and immunoprecipitates of VE-cadherin or Tie-2 from lung lysates were analyzed for phospho-tyrosines in immunoblots. As shown in Fig. 4 (B and C), the rapalog decreased tyrosine phosphorylation of VE-cadherin,  $\beta$ -catenin and plakoglobin, but not of Tie-2. As an additional control, we tested whether the rapalog or the expression of VE-cadherin-FKBP affected the distribution of cortical actin or myosin light chain (MLC)-2 in primary endothelial cells isolated from lungs of the KI mice. As shown in Fig. S3, we found no such effects.

Because *i.v.* injection of VEGF did not induce plasma protein leakage into the alveolar space, probably because of a lack of effect on the alveolar epithelium, we used the Miles permeability assay in the skin. In this way, we tested whether preventing the dissociation of VE-PTP from VE-cadherin would impair the induction of vascular permeability by VEGF *in vivo*. We injected KI mice *i.v.* with 250  $\mu$ g rapalog or vehicle 8 and 4 h before the assay. Subsequently, mice were *i.v.* injected with Evan's blue dye and stimulated intradermally 10 min later with VEGF or PBS. Injected skin areas were excised 30 min later, and the dye was extracted with formamide.



**Figure 4. Dissociation of VE-PTP from VE-cadherin is necessary for VEGF-induced permeability *in vivo*.** (A) Homozygous KI mice were injected *i.v.* either with vehicle alone (left) or with rapalog (right) 8 and 4 h before the assay. Mice were then *i.v.* injected with either PBS or with VEGF (as indicated), and 30 min later lung lysates were immunoprecipitated for VE-PTP-FRB\*. Precipitated material was analyzed by immunoblotting for VE-cadherin-FKBP and VE-PTP expression. To control for degradation, aliquots of total lung lysates were set aside and analyzed by immunoblotting for VE-cadherin-FKBP (bottom). The amount of immunoprecipitated VE-PTP-FRB and co-precipitated VE-cadherin-FKBP detected from VEGF-treated mice is shown as percentage of the amount precipitated from PBS-treated mice (numbers below). Graphs in inserts give co-precipitation efficiency. Note that the rapalog blocked dissociation of VE-cadherin from VE-PTP. (B) Homozygous KI mice were treated with rapalog or vehicle and subsequently with VEGF as in A. Lung lysates were immunoprecipitated for VE-cadherin and precipitates were immunoblotted first for phosphotyrosine, and then for VE-cadherin and plakoglobin ( $\gamma$ -catenin). (C) Lung lysates from KI mice were immunoprecipitated for Tie-2 and immunoblots were analyzed for phospho-tyrosine and subsequently for Tie-2. (D) Homozygous KI mice were injected *i.v.* with rapalog (black bars) or vehicle alone (white bars) 8 and 4 h before the assay. At the start of the assay, Evan's blue was *i.v.* injected, followed by intradermal injection of VEGF or PBS 10 min later. After 30 min, mice were sacrificed and the dye was extracted from skin samples and quantified.  $n = 5$  mice per group. Data are representative of two (A–C) or three (D) independent experiments. (E) Homozygous KI mice were treated with rapalog or vehicle as in D, followed by exposing the mice to nebulized LPS for 45 min. 4 h later, lungs were lavaged and protein content was measured.  $n = 3$  mice per group. Results shown are representative of two independent experiments. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ .



**Figure 5. Dissociation of VE-PTP from VE-cadherin is necessary for efficient transendothelial migration of leukocytes in vivo.** Homozygous KI mice were i.v. injected with either rapalog or vehicle alone 8 and 4 h before the assay, followed by an intrascrotal injection of 50 ng IL-1 $\beta$ . The cremaster muscle was prepared 4 h later for intravital imaging. Numbers of extravasated (A) and adherent leukocytes (B), rolling flux fraction (C), and rolling velocity (D) were determined. The results are displayed as mean  $\pm$  SEM of at least 30 vessels from five independent animals in each group. (E) Homozygous KI mice were treated with rapalog or

As shown in Fig. 4 D, VEGF induced a 2.5-fold increase in vascular permeability in vehicle-treated KI mice, whereas VEGF no longer induced any significant increase in permeability in rapalog-treated mice. No such inhibitory effect was seen with the rapalog in WT mice (not depicted) or in mice in which exon 2 of VE-cadherin had been replaced by the cDNA for WT VE-cadherin (Fig. S4).

Analyzing an alternative model of LPS-induced pulmonary inflammation, we found that the rapalog inhibited most of the LPS-induced plasma protein leak into the alveolar space (Fig. 4 E). Thus, the dissociation of VE-PTP from VE-cadherin is indeed a necessary step in the molecular process whereby VEGF or LPS stimulates vascular permeability *in vivo*.

#### Dissociation of VE-PTP from VE-cadherin is necessary for efficient leukocyte diapedesis *in vivo*

Next, we wanted to know whether the dissociation of VE-PTP from VE-cadherin is necessary for efficient leukocyte diapedesis and extravasation in inflammation. To test this, we analyzed leukocyte extravasation by intravital microscopy of the IL-1 $\beta$ -stimulated cremaster in our KI mice. The mice were pretreated with rapalog or vehicle as described for Fig. 4, and mice were intrascrotally stimulated with IL-1 $\beta$  immediately after the second injection. 4 h later, the cremaster muscle was analyzed by intravital microscopy. We found that IL-1 $\beta$ -induced extravasation of leukocytes was reduced by  $\sim$ 50% by the rapalog (Fig. 5 A), whereas neither the number of adhering or rolling leukocytes nor the rolling velocity were significantly affected (Fig. 5, B–D). In addition, peripheral leukocyte counts were unaffected by the rapalog (unpublished data). Importantly, the rapalog had no effect on IL-1 $\beta$ -induced leukocyte extravasation in WT mice (not depicted) or in mice in which exon 2 of VE-cadherin had been replaced by a WT VE-cadherin cDNA (Fig. S4).

As an additional inflammation model, we analyzed LPS-induced acute lung injury. KI mice were exposed for 45 min to nebulized LPS upon rapalog administration, and 4 h later lungs were lavaged and infiltrated neutrophils were counted. As shown in Fig. 5 E, rapalog inhibited neutrophil recruitment by 57%. We conclude that the dissociation of VE-PTP from VE-cadherin is indeed required for efficient leukocyte extravasation in the inflamed cremaster and the lung.

Our results demonstrate for the first time that the association of VE-PTP with VE-cadherin stabilizes endothelial cell contacts *in vivo*. We show that LPS-induced inflammation and systemic stimulation with VEGF induce the dissociation of this complex *in vivo*. Preventing this dissociation inhibits LPS- and cytokine-induced leukocyte extravasation and blocks

vehicle and exposed to nebulized LPS as in Fig. 4 E. The leukocytes in the bronchoalveolar lavage fluid were counted and the numbers of neutrophils were determined by FACS.  $n = 3$  mice per group. Results shown are representative for two independent experiments. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ .

VEGF- and LPS-induced vascular permeability. Thus, we show here that VE-PTP is indeed crucial for the pathophysiological regulation of endothelial cell contacts *in vivo*.

This is the first *in vivo* evidence demonstrating that tyrosine phosphorylation of components of the VE-cadherin–catenin complex, or of proteins associated with this complex and within reach of VE-cadherin-associated VE-PTP, is an essential step during the opening of endothelial junctions in both processes. This establishes VE-PTP as an attractive target for the treatment of pathological vascular permeability and inflammation.

## MATERIALS AND METHODS

**Cell culture and reagents.** COS-7 cells were cultured as described previously (Fachinger et al., 1999). Transient transfections were performed using the GeneJammer transfection reagent (Stratagene Europe) according to the manufacturer's instructions. Cells were analyzed 24 h after transfection. Rapalog (AP21967) was provided by ARIAD Pharmaceuticals, Inc. Lyophilized rapalog was diluted in ethanol to prepare a 62.5 mg/ml stock solution. Mice were injected with freshly prepared rapalog using 4% of the rapalog stock solution, 10% PEG-400 (Sigma-Aldrich) in 1.7% Tween-20 (Merck) in dH<sub>2</sub>O. Primary endothelial cells from lungs of WT or KI mice were isolated and cultured as previously described (Schulte et al., 2011).

**Antibodies.** The following antibodies were used: pAb VE42 and mAb 11D4.1 against mouse VE-cadherin (Gotsch et al., 1997); mAb BV-13 against mouse VE-cadherin (Abcam); mAb against  $\beta$ -catenin, mAb against plakoglobin, mAb against  $\alpha$ -catenin and mAb against p120-catenin (all from BD); mAb 4G10 against phosphotyrosine (Millipore); pAb PTP 1–8 against the extracellular fibronectin type III-like domains 1–8 of VE-PTP; pAb VE-PTP-C against VE-PTP (Nawroth et al., 2002); mAb 3G1 against Tie-2 (Koblizek et al., 1997); mAb and pAb against Flag-tag (Sigma-Aldrich); pAb against HA-tag (Abcam); pAb against MLC-2 (Cell Signaling Technology); and Phalloidin-Alexa Fluor 568 (Invitrogen).

**Constructing fusion proteins.** VE-cadherin-FKBP and Flag-VE-PTP-FRB\* (a shortened form of VE-PTP containing an extracellular, N-terminal Flag-tag and the 17th FNIII-like repeat) were generated by PCR amplification of VE-cadherin from pCDNA3-VE-cadherin (Nottebaum et al., 2008) and Flag-VE-PTP were generated by PCR amplification of pFlagCMV1-VE-PTP (Fachinger et al., 1999) using primers containing an XbaI site. For VE-cadherin amplification, VE-cad-XbaI-S (5'-GTATCTAGAAGTGCAGTGGAGAGAGGCC-3') and VE-cad-XbaI-AS (5'-GCGTCTAGAGATGATGAGTTCCTCCTG-3') were used. For Flag-VE-PTP amplification, VE-PTP-XbaI-S (5'-CGTCTAGAGAGCTCGTTTGTAGTGAACC-3') and PTP-XbaI-AS (5'-CGTCTAGAATGTCTCGAGTAGATTGC-3') were used. VE-cadherin and Flag-VE-PTP were cloned via the XbaI sites into pC4EN-F1 and pC4-RHE (ARIAD Pharmaceuticals, Inc). VE-cadherin-FKBP was also inserted into pCDNA3-VE-cadherin via EcoNI, and HindII/EcoRV and Flag-VE-PTP-FRB\* was inserted into pGeneV5HisC-VE-PTP (Nottebaum et al., 2008) via ClaI and MfeI/BstBI.

**Generation of KI mice.** VE-cadherin-FKBP and full-length VE-PTP-FRB\* were expressed in mice by replacing endogenous VE-cadherin by cDNAs for the two fusion proteins, using RMCE. To this end, the two cDNAs, separated by an IRES site were cloned into a cassette exchange vector, by inserting VE-cadherin-FKBP into the MCS A and full-length VE-PTP-FRB\* into MCS B of the pIRES vector (Takara Bio Inc.). For subsequent ES cell transfection, the two cDNAs flanking the IRES site were inserted into the cassette exchange vector U5-3, containing a polyA transcriptional stop cassette and a hygromycin cassette flanked with FRT sites. The complete insertion cassette was flanked by the two incompatible recombination sites LoxP and Lox2272. For RMCE, exon 2 of VE-cadherin containing the start codon was flanked with a LoxP and a Lox2272 site by homologous

recombination of mouse ES cells using the targeting vector U2-HR that contained a 6.9-kb long arm 5' of exon 2 and the LoxP/Lox2272-flanked exon 2 of VE-cadherin, followed by an FRT-flanked neo cassette and a 1.5-kb short arm. Neomycin-resistant clones were screened by PCR and Southern blot analysis. For RMCE, positive clones were co-transfected with the aforementioned RMCE exchange vector and a Cre recombinase expression plasmid. Hygromycin-resistant colonies were screened by PCR and confirmed by Southern blotting. Positive ES cell clones were injected into blastocysts of C57BL/6 mice to generate chimeras, which were mated with C57BL/6 mice. The generated mice were intercrossed to generate homozygous VE-cadherin FKBP/VE-PTP FRB\* mice. For controls mice were generated by inserting a VE-cadherin cDNA in the same way into the VE-cadherin locus. Genotyping was performed using primers RMCEfor (5'-GAAGAGCTTTCGGGCTGGAATGACC-3') and RMCErev (5'-GGATGATGGTAGCAGGTGTGGGG-3'), generating a 520-bp PCR product in WT mice and a 565-bp PCR product in KI mice. Homozygous KI mice developed normally and were viable.

All animal experiments were approved by the Animal Care and Use Committees of the University of Münster (Germany) and the local government. Animals were kept in a barrier facility under special pathogen-free conditions.

**Immunoprecipitation and immunoblotting.** For co-immunoprecipitations cells were lysed in lysis buffer containing (20 mM Imidazole, pH 6.8, 100 mM NaCl, 2 mM CaCl<sub>2</sub>, 1% Triton X-100, 0.04% NaN<sub>3</sub>, and 1X Complete EDTA-free protease inhibitor cocktail [Roche]). For detection of phosphotyrosine after immunoprecipitation, cells were lysed in lysis buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM CaCl<sub>2</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton X-100, 0.04% NaN<sub>3</sub>, and 1X Complete EDTA-free. For co-immunoprecipitations from mouse lung lysates, lungs were homogenized with an Ultra Turrax (IKA-Werke) in three parts of PBS containing 1 mM CaCl<sub>2</sub> and 2X Complete EDTA-free protease inhibitor cocktail. Subsequently, one part of 4X lysis buffer (PBS, 4% NP-40, 4 mM dithiothreitol [DTT], and 1 mM CaCl<sub>2</sub>) was added and incubated over night at 4°C. Lysates were centrifuged at 4°C for 30 min or 1 h for lung lysates at 20,000 g, aliquots were set aside for direct blot analysis, and aliquots for immunoprecipitation were incubated for 2 h at 4°C with protein A-Sepharose loaded with the respective antibodies. Immunocomplexes were washed five times with lysis buffer and analyzed by SDS-PAGE. In some cases, mice received an i.v. injection of 3  $\mu$ g VEGF<sub>165</sub> (PeproTech) in 100  $\mu$ l PBS, or were exposed for 1 h to nebulized LPS from Sigma-Aldrich (3.5 mg in 7 ml 0.9% NaCl) before preparation of the lung lysates. Control mice received PBS only. Total cell lysates, organ lysates, or immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose (Schleicher & Schuell) by wet blotting. Blots were analyzed as previously described (Ebnet et al., 2000). In some cases blots were quantified using the luminescent image analyzer LAS-4000 (Fuji Film) and the software MultiGauge V3.2 (Fuji Film). For detection of phosphotyrosine, milk powder in the blocking buffer was replaced by 2% BSA, and 200  $\mu$ M Na<sub>3</sub>VO<sub>4</sub> was added.

**Immunofluorescence staining.** Primary murine endothelial cells isolated from lungs were grown to confluency on laminin-coated transwell filters. Cells were fixed using 2% PFA/PBS. Primary antibodies were detected using Alexa Fluor 488- or Alexa Fluor 633-coupled secondary antibodies (Invitrogen). Fluorescence signals were detected using a confocal laser scanning microscope (LSM 780 inverted microscope; Carl Zeiss, Inc.). Cremaster whole mount stainings were done as previously described (Schulte et al., 2011).

**In vivo permeability assay.** Mice were injected i.v. with rapalog (250  $\mu$ g per injection) or with vehicle 8 and 4 h before the assay. Modified Miles assay for VEGF<sub>165</sub>-stimulated (PeproTech) vascular permeability in the skin was described previously (Mamluk et al., 2005). For each assay, 3 to 5 8–12-wk-old KI and 3 to 5 sex-matched wild-type KI mice were used. In brief, Evan's blue dye (Sigma-Aldrich) was injected into the tail vein (100  $\mu$ l of a 1% solution in PBS), and 10 min later, 50  $\mu$ l PBS or 100 ng mouse VEGF<sub>165</sub> in 50  $\mu$ l PBS was intradermally injected into the shaved back skin. 30 min later, skin areas

were excised and extracted with formamide for 5 d and the concentration of the dye was measured at 620 nm in a spectrophotometer (Shimadzu).

**Intravital microscopy.** 8 and 4 h before the assay, mice were injected with vehicle or rapalog (250  $\mu\text{g}$  per injection). Directly after the second rapalog/vehicle injection mice received an intrascrotal injection of 50 ng IL-1 $\beta$  (R&D Systems) in 0.3 ml of saline. 4 h later, mice were anesthetized using i.p. injection of 125 mg/kg ketamine hydrochloride (Sanofi), 12.5 mg/kg xylazine (Tranqui Ved), and 0.025 mg/kg atropine sulfate (Fujisawa), and the cremaster muscle was prepared for intravital imaging as previously described (Zarbock et al., 2007, 2008). Intravital microscopy was performed on an upright microscope (Axioskop; Carl Zeiss, Inc.) with a 40  $\times$  0.75 NA saline immersion objective. Leukocyte rolling velocity, leukocyte rolling flux fraction, and leukocyte arrest were determined by transillumination intravital microscopy, whereas leukocyte extravasation was investigated by reflected light oblique transillumination microscopy as previously described (Mempel et al., 2003). Recorded images were analyzed off-line using ImageJ and AxioVision (Carl Zeiss, Inc.) software. Leukocyte rolling flux fraction was calculated as percentage of total leukocyte flux. Emigrated cells were determined in an area reaching out 75 mm to each side of a vessel over a distance of 100-mm vessel length (representing  $1.5 \times 10^4$  mm<sup>2</sup> tissue area). The microcirculation was recorded using a digital camera (Sensicam). Postcapillary venules with a diameter between 20–40  $\mu\text{m}$  were investigated. Blood flow centerline velocity was measured using a dual-photodiode sensor system (Circusoft).

**Murine model of LPS-induced pulmonary inflammation.** 8 and 4 h before the assay, mice were vehicle or rapalog injected (125  $\mu\text{g}$  per injection). LPS-induced pulmonary inflammation was achieved as previously described (Zarbock et al., 2009). In brief, LPS from 500  $\mu\text{g}/\text{ml}$  *Salmonella enteritidis* (Sigma-Aldrich) was nebulized and mice were exposed for 45 min to nebulized LPS or saline as a control. 4 h later, mice were sacrificed and the lungs were lavaged with PBS. After centrifugation, the protein concentration in the supernatant was determined using the BCA Protein Assay kit (Thermo Fisher Scientific). Leukocyte counts in the bronchoalveolar lavage fluid were analyzed and the numbers of neutrophils were determined by FACS.

**Statistical analysis.** Datasets were checked for normality (Shapiro-Wilk) and equal variance. When possible P values were determined by Student's *t* test. Otherwise Mann-Whitney Rank Sum Test was applied. Analysis was done using SigmaPlot 10.0. Error bars indicate standard error of mean values.

**Online supplemental material.** Fig. S1 shows genotyping results for the KI mice and the respective ES cells. Fig. S2 shows that antibodies against VE-cadherin can remove VE-cadherin-FKBP as efficiently as WT VE-cadherin from cell contacts of primary endothelial cells from lungs of the analyzed KI mice. Fig. S3 shows that neither application of rapalog nor the expression of VE-cadherin-FKBP alters actin organization. Fig. S4 shows that the rapalog does not inhibit permeability induction or leukocyte recruitment in WT mice.

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