Diminution of signal transducer and activator of transcription 3 signaling inhibits vascular permeability and anaphylaxis

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Background: During IgE-mediated immediate hypersensitivity reactions, vascular endothelial cells permeabilize in response to mast cell mediators. We have demonstrated previously that patients and mice with signal transducer and activator of transcription 3 *(STAT3)* mutations (autosomal dominant hyper-IgE syndrome [AD-HIES]) are partially protected from anaphylaxis.

Objectives: We sought to study the mechanism by which STAT3 contributes to anaphylaxis and determine whether small-molecule inhibition of STAT3 can prevent anaphylaxis.

Methods: Using unaffected and STAT3-inhibited or genetic loss-of-function samples, we performed histamine skin prick tests, investigated the contribution of STAT3 to animal models of anaphylaxis, and measured endothelial cell permeability, gene and protein expression, and histamine receptor–mediated signaling.

Results: Although mouse mast cell degranulation was minimally affected by STAT3 blockade, mast cell mediator–induced anaphylaxis was blunted in *Stat3* mutant mice with AD-HIES and in wild-type mice subjected to small-molecule STAT3 inhibition. Histamine skin prick test responses were diminished in patients with AD-HIES. Human umbilical vein endothelial cells derived from patients with AD-HIES or treated with a STAT3 inhibitor did not signal properly through Src or cause appropriate dissolution of the adherens junctions made up of the proteins vascular endothelial–cadherin and β-catenin. Furthermore, we found that diminished STAT3 target microRNA17-92 expression in human umbilical vein endothelial cells from patients with AD-HIES is associated with increased phosphatase and tensin homolog (PTEN) expression, which inhibits Src, and increased E2F transcription factor 1 expression, which regulates β-catenin cellular dynamics.

Conclusions: These data demonstrate that STAT3-dependent transcriptional activity regulates critical components for the architecture and functional dynamics of endothelial junctions, thus permitting vascular permeability. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: *Allergy, immunology, innate immunity, signal transducer and activator of transcription 3, autosomal dominant hyper-IgE syndrome*

The signal transducer and activator of transcription (STAT) family of transcription factors are integral components of many cytokine receptor signaling systems.[1-3] Multiple clinical phenotypes result from both germline gain- and loss-of-function mutations in STATs.[4-7] Dominant negative *STAT3* mutations in human subjects result in dermatitis, increased serum IgE levels, enhanced susceptibility to staphylococcal skin and respiratory tract infection, mucocutaneous candidiasis, and connective tissue and skeletal abnormalities.[8]

Despite a significant burden of eczematous skin disease and associated increases in both total and allergen-specific serum IgE levels, clinical food allergy and anaphylaxis are markedly diminished in patients with autosomal dominant hyper-IgE syndrome (AD-HIES).[9] One potential mechanism contributing to this phenomenon might involve STAT3-mediated regulation of mast cell degranulation[9] and mitochondrial activity.[10]

After mast cell degranulation, mediators, such as histamine, platelet-activating factor (PAF), and thrombin, act on target vascular endothelium to induce synthesis of nitric oxide (a potent vasodilator),[11,12] intracellular calcium release,[13,14] and vascular leak, resulting in symptoms of immediate hypersensitivity allergic reactions, including flushing and hypotension.[15,16] Factors, such as histamine, PAF, or vascular endothelial growth factor, result in destabilization of vascular endothelial cadherin (VE-cadherin) in the adherens junctions[17] by uncoupling VE-cadherin from β-catenin anchors through a Src/Yes kinase–dependent mechanism.[18-20]

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Abbreviations used
AD-HIES: Autosomal dominant hyper-IgE syndrome
BMMC: Bone marrow–derived mast cell
DMSO: Dimethyl sulfoxide
DNP-HSA: Dinitrophenyl–human serum albumin
E2F1: E2F transcription factor 1
FITC-Dex: Fluorescent isothiocyanate–dextran
HUVEC: Human umbilical vein endothelial cell
MCPT-1: Mast cell protease 1
mir17-92: MicroRNA17-92
MLE: Mouse lung endothelial
PAF: Platelet-activating factor
PDMC: Peritoneal cell–derived mast cell
PTEN: Phosphatase and tensin homolog
SIAH1: Siah E3 ubiquitin protein ligase 1
STAT: Signal transducer and activator of transcription
VE-cadherin: Vascular endothelial cadherin
WT: Wild-type

STAT3 is activated after adherens junctions formation,[21] and STAT3 signaling has been implicated in gap junction intercellular communication, IL-6–induced vascular leakage, downregulation of VE-cadherin, and microRNA17-92 (mir17-92)/E2F1–dependent regulation of β-catenin nuclear translocation and transcriptional activity.[22-28] However, the specific role of STAT3 in endovascular permeability, in particular to mast cell mediators, has not been explored. Here we demonstrate that intact STAT3 signaling is essential for mast cell mediator–induced vascular endothelial permeability and that small-molecule inhibition of STAT3 prevents endothelial permeability *in vitro* and anaphylaxis *in vivo*.

METHODS

A full description of the methods used in this study can be found in the Methods section in this article's Online Repository at www.jacionline.org.

Histamine skin prick tests

Histamine skin prick tests responses were measured at 15 minutes and recorded per the standard of care. Data are reported as the total area calculated by the length equating to the widest point of a wheal and flare multiplied by the width measuring perpendicular to the widest point.

Mice and STAT3 inhibition *in vivo*

Wild-type (WT) C57Bl/6 mice were obtained from the Jackson Laboratory (Bar Harbor, Me), and mut-*Stat3* (AD-HIES) mice with their corresponding WT littermate control animals were kindly provided by Dr J. O'Shea.[29] Mice received daily intraperitoneal injections with either C188-9 at 50 mg/kg or vehicle for 1, 4, or 7 days. Anaphylaxis was induced 24 hours after the last C188-9 injection.

Mouse model of systemic anaphylaxis

Systemic anaphylaxis was measured, as previously described.[30] For IgE-induced passive systemic anaphylaxis, WT mice were sensitized intravenously with 3 μg of DNP-specific IgE (200 μL, clone H1-DNP-ε-26.82) and challenged 24 hours later with 200 μg of dinitrophenyl–human serum albumin (DNP-HSA; administered intravenously; Sigma-Aldrich, St Louis, Mo). Alternatively, systemic anaphylaxis was induced in WT mice or mice with AD-HIES by an intravenous bolus of histamine dihydrochloride (5 μmol in 200 μL of PBS, Sigma-Aldrich) or by 0.3 μg of PAF (Tocris Bioscience, Bristol, United Kingdom).

For measurement of histamine and mast cell protease 1 (MCPT-1) levels released into the circulation, C188-9- and vehicle-treated mice were killed by means of CO_2 inhalation 90 seconds or 30 minutes after induction of anaphylaxis. Blood was collected in EDTA-containing tubes (BD Biosciences, San Jose, Calif), and histamine and MCPT-1 levels in plasma were measured with specific ELISA kits (Beckman Coulter, Fullerton, Calif, and eBioscience, San Diego, Calif, respectively).

Assessment of murine vascular permeability *in vivo*

Hematocrit levels were determined by collecting blood from the retro-orbital plexus 2 days before (baseline) and 2 hours after induction of anaphylaxis in C188-9- and vehicle-treated mice, with heparinized microhematocrit tubes (Jorvet, Loveland, Colo), and the volume percentage of red blood cells was determined with a hematocrit reader.

After sensitization, mice were challenged intravenously 24 hours later with 200 μg of DNP-HSA in 200 μL of PBS containing 1% Evans Blue dye (Sigma-Aldrich) to investigate peripheral vascular leakage in response to IgE/antigen-induced anaphylaxis in the skin. Evans Blue dye extravasated into the ear tissue was extracted with 700 μL of formamide at 55°C for 2 hours and quantified by measuring absorbance at 620 nm after clearing the extracts by using centrifugation.

Cell culture

Cell-culture conditions used in this study can be found in the Methods section in this article's Online Repository.

In vitro inhibitor treatments

For *in vitro* treatments, C188-9 was added to the cells at a concentration of 1 μmol/L, unless stated otherwise. The optimal dose was determined to be 1 μmol/L after performing a dose-response assay of 500 nm, 1 μmol/L, and 10 μmol/L. For phosphatase and tensin homolog (PTEN) inhibitor treatment, VO-OHpic (Echelon Biosciences, Salt Lake City, Utah) was added to the cells at a concentration of 250 to 500 nmol/L. All inhibitors were compared with dimethyl sulfoxide (DMSO)–treated controls.

Mast cell degranulation assays

LAD2 cells, 7- to 10-week-old primary human mast cells, 4- to 6-week-old bone marrow–derived mast cells (BMMCs), or peritoneal cell–derived mast cell (PDMCs) were incubated with different concentrations of C188-9 (0.3-10.0 μmol/L) or DMSO for 1 week. Degranulation was measured as β-hexosaminidase release into the medium 30 minutes after antigen challenge (100 ng/mL DNP-HSA, Sigma-Aldrich) for DNP-IgE–sensitized murine mast cells or after 10 ng/mL streptavidin (Sigma-Aldrich) for biotinylated IgE-coated human mast cells, as previously described.[31]

Flow cytometry

After 7 injections of C188-9 (or vehicle), mice were killed, and peritoneal lavage fluid was obtained by using 10 mL of RPMI media and stained with an antibody specific for phosphorylated STAT3-Serine727-PE (catalog no. 558557; BD Biosciences, San Jose, Calif).

LAD2 cells were serum starved in RPMI for 30 minutes at 37°C and then treated with C188-9 (3, 30, and 100 μmol/L) for 1 hour at 37°C. Samples were stimulated with 100 ng/mL IL-6 for 15 minutes or 500 ng/mL IFN-γ for 15 minutes and then fixed and permeabilized (as described in the Methods section in this article's Online Repository). After washing with FACS buffer, cells were stained with phosphorylated STAT1–Alexa Fluor 488 (catalog no. 612596; BD Biosciences) and phosphorylated STAT3–tyrosine 705–Alexa Fluor 647 (catalog no. 557815, BD Biosciences) for 30 minutes.

Immunofluorescence

Primary antibodies used were total β-catenin (catalog no. 2677; 1:100), active (nonphosphorylated) β-catenin (catalog no. 8814; 1:100), and VE-cadherin (catalog no. 2500; 1:100; Cell Signaling, Danvers, Mass). For full details, see the Methods section in this article's Online Repository.

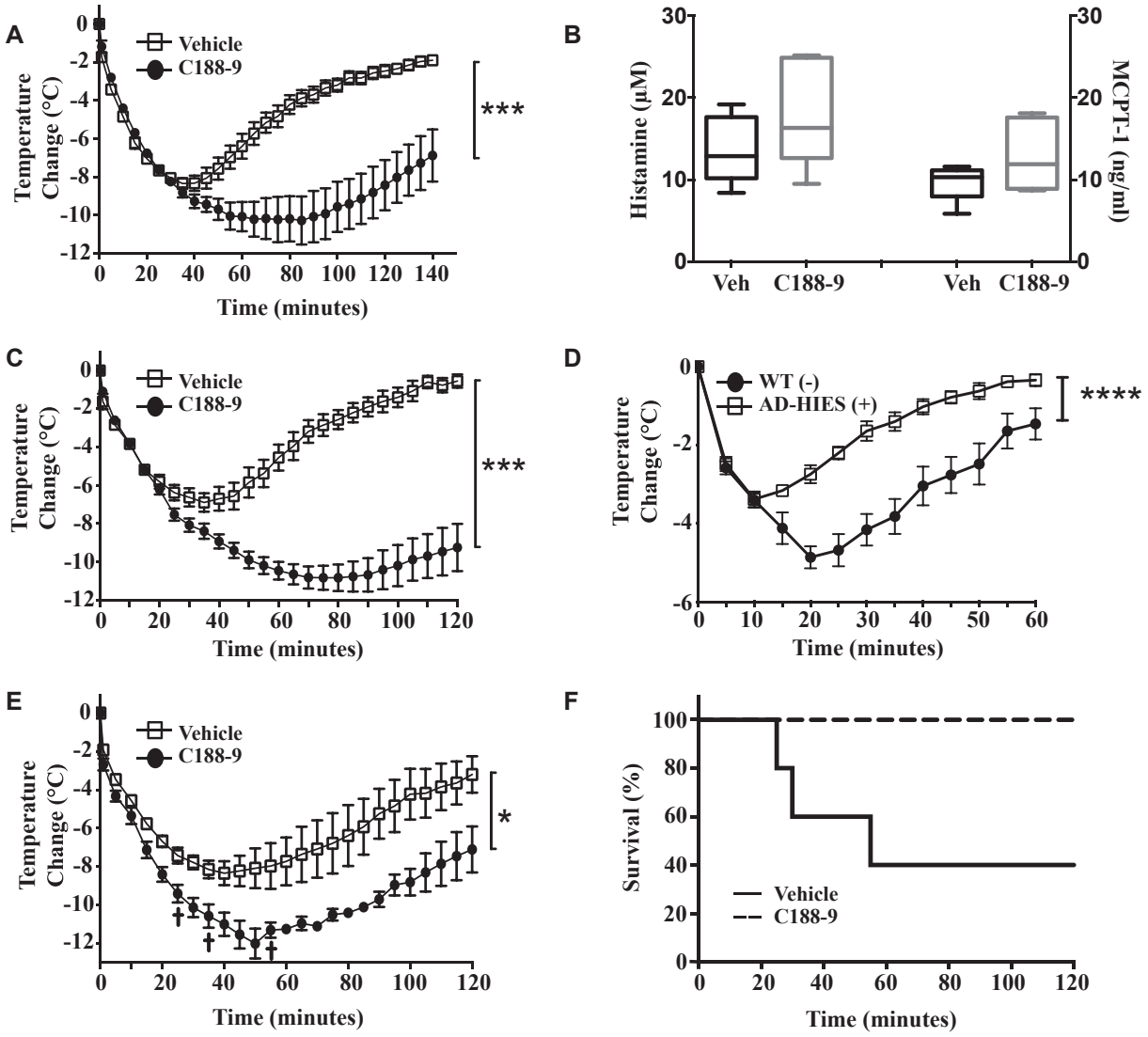


FIG 1. Reduction in STAT3 levels confers resistance to anaphylaxis. **A,** Effect of C188-9 pretreatment (7 days) on body temperature changes in male C57Bl/6 mice during anaphylactic shock. Mice (n = 5-6 per group) were sensitized with 3 μg of DNP-specific IgE and challenged 24 hours later with 200 μg of DNP-HSA. **B,** Histamine and MCPT-1 released into the circulation (90 seconds) after challenge sensitized C188-9- or vehicle-treated mice to antigen (n = 6 per group). Results are presented as box plots with minimum/maximum range. **C** and **D,** Temperature changes during anaphylaxis induced by histamine (5 μmol) in C188-9- and vehicle-treated C57BL/6 (Fig 1, *C*) or AD-HIES (Fig 1, *D*) mice (n = 6 per group). **E,** Temperature changes during anaphylaxis induced by PAF (0.3 mg) in C188-9- and vehicle-treated mice. **F,** Survival curve of mice after PAF treatment (n = 6 per group in Fig 1, *B*, and n = 5 per group in Fig 1, *C*). $*P < .05$, $***P < .001$, and $****P < .0001$.

Real-time PCR

Predesigned TaqMan primers and probes were purchased from Life Technologies (Grand Island, NY) and real-time PCR was performed with a 7500 real-time PCR system (Life Technologies).

Transfections

Human umbilical vein endothelial cells (HUVECs) were transfected with the Nucleofector transfection system (Lonza, Allendale, NJ), according to the manufacturer's recommended protocol. Briefly, 5×10^5 cells were resuspended in Nucleofector solution; combined with 200 nmol/L of either a nontargeting microRNA mimic, a mir19a microRNA mimic, a nontargeting antagomir, or an mir19a antagomir (Dharmacon, Lafayette, Colo); and delivered to the intracellular environment by means of electroporation. Cells were then added to pre-equilibrated culture medium for 48 hours and then underwent a second transfection. Finally, 24 hours later, cells were harvested for Western analysis or seeded for permeability assays.

Cytoplasmic/nuclear extraction

Cytoplasmic and nuclear cell fractions were isolated by using the NE-PER kit (Thermo Fisher, Rockford, Ill), according to the manufacturer's protocol, and then analyzed by using SDS-PAGE and Western blotting.

SDS-PAGE and Western blotting

Fifty micrograms of protein was isolated and subjected to SDS-PAGE and transferred onto polyvinylidene difluoride. Primary antibodies (see the

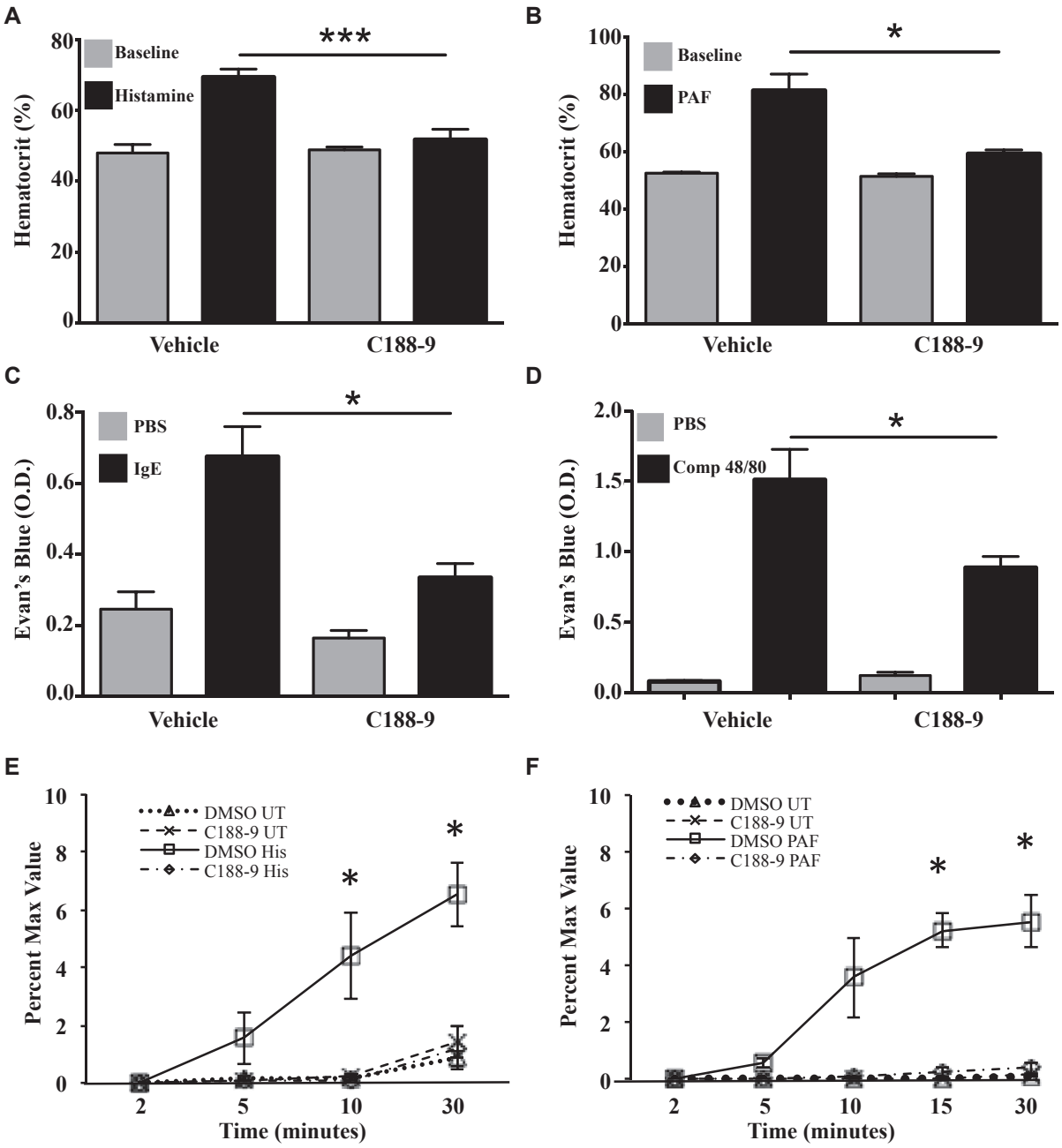


FIG 2. C188-9 decreases histamine- or PAF-induced vascular leakage. **A** and **B,** Hematocrit values at baseline and after passive systemic anaphylaxis induced by histamine (Fig 2, *A*) or PAF (Fig 2, *B*) in C188-9-treated (n = 5 per group) and vehicle-treated (n = 6 per group) mice. **C** and **D,** Evans Blue dye leakage in response to IgE/antigen c (n = 10 per group; Fig 2, *C*) or compound 48/80 (n = 5 per group; Fig 2, *D*) challenge in C188-9- and vehicle-treated mice. **E** and **F,** *In vitro* permeability assay of mouse lung endothelial cells exposed to histamine (1 μmol/L; Fig 2, *E*) or PAF (10 μg; Fig 2, *F*) after pretreatment with DMSO or C188-9 (1 μmol/L, n = 3 per group). *His,* Histamine; *UT,* untreated. Data are representative of 3 independent experiments and expressed as means ± SEMs. **P* < .05 and ****P* < .001.

Methods section in this article's Online Repository) were added at appropriate dilutions in 5% milk/Tris-buffered saline–Tween 20 and incubated overnight at 4 °C with rotation. Membranes were subsequently washed 3 times in PBS, and horseradish peroxidase–conjugated secondary antibodies were added at 1:2000 for 1 hour at room temperature (Santa Cruz Biotechnology, Santa Cruz, Calif). Bands were visualized with ECL Plus (Thermo Fisher).

Calcium assays

WT HUVECs, AD-HIES HUVECs, and mouse lung endothelial (MLE) cells were seeded at 20,000 cells/well in a 96-well plate (Corning, Tewksbury, Mass) and incubated overnight. A Fluo-8 calcium assay was then performed (Abcam, Cambridge, Mass). Cells were washed in PBS, and 100 μL of Fluo-8 dye loading solution was added to each well. Plates were incubated at 37°C for 30 minutes and then for 30 minutes at room temperature. Histamine was added

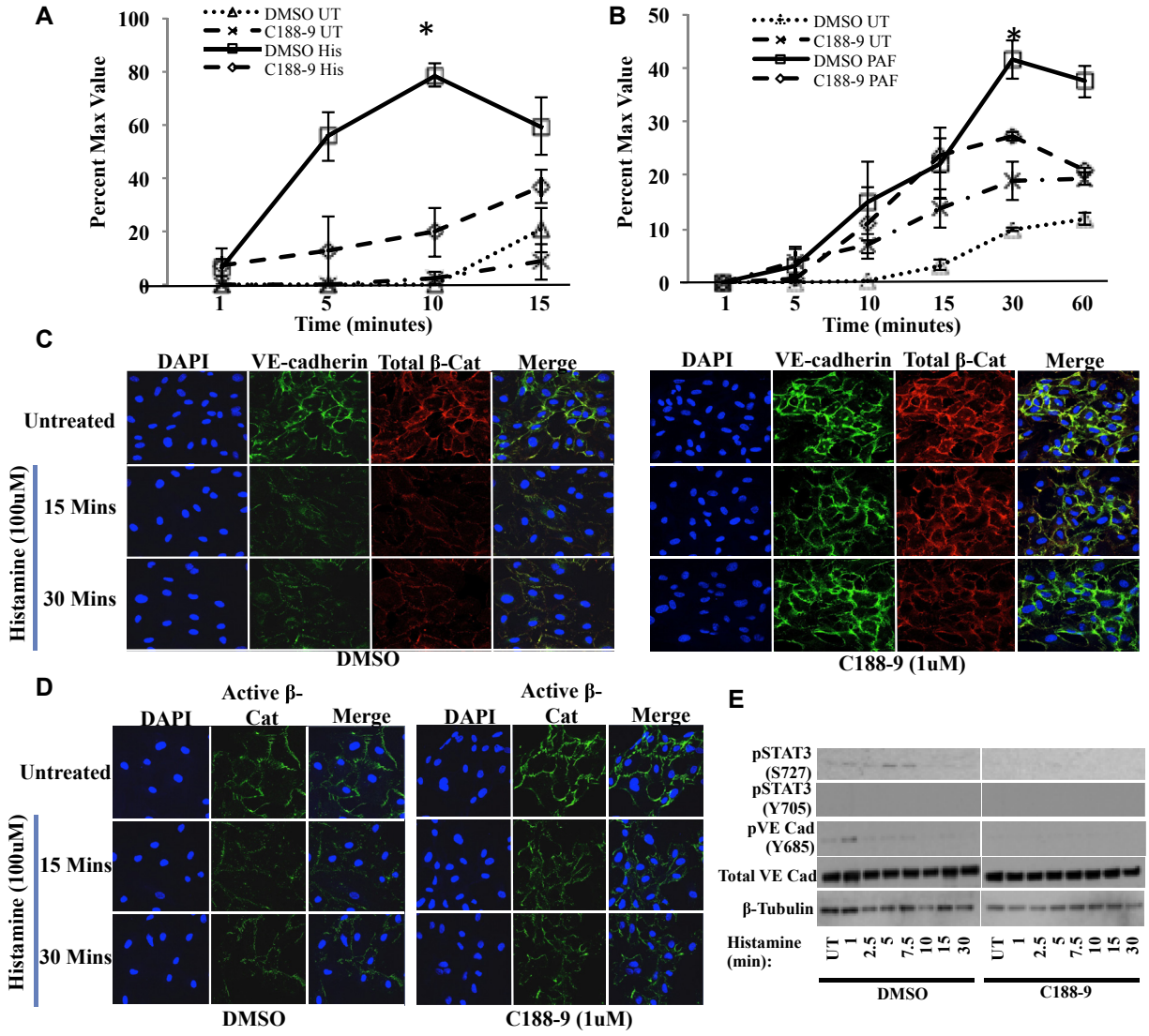


FIG 3. C188-9 decreases vascular permeability through strengthening VE-cadherin junctions. **A** and **B,** *In vitro* permeability assay of HUVECs pretreated with DMSO or C188-9 (1 μmol/L) and subsequently exposed to histamine (100 μmol/L; Fig 3, *A*) or PAF (10 μg/mL; Fig 3, *B*). *His,* Histamine; *UT,* untreated. Data are representative of 3 independent experiments. **C,** Confocal microscopy of VE-cadherin (green), total β-catenin (red), and 4′-6-diamidino-2-phenylindole hydrochloride (*DAPI*; blue) in DMSO- and C188-9 (1 μmol/L)–pretreated HUVECs then subsequently exposed to histamine (100 μmol/L). **D,** Confocal microscopy of active (nonphosphorylated) β-catenin (green) and DAPI (blue) in DMSO- and C188-9 (1 μmol/L)–pretreated HUVECs then subsequently exposed to histamine (100 μmol/L). **E,** Western analysis of Stat3 and VE-cadherin signaling in DMSO- and C188-9–treated HUVECs. Data are expressed as means ± SEMs. $*P < .05$.

(1, 10, 100, and 1000 μmol/L), and fluorescent intensity was monitored at an excitation/emission (Ex/Em) value of 490/525 nm by using a FlexStation II (Molecular Devices, Sunnyvale, Calif).

In vitro permeability assays

In vitro permeability assays were performed as previously described[32,33] and in the Methods section in this article's Online Repository. Cells were exposed to 1 mg of fluorescent isothiocyanate–dextran (FITC-Dex; Sigma) and histamine, PAF, or IL-11. After collecting all time points, fluorescent intensity was monitored at an Ex/Em value of 490/525 nm.

STAT3 reporter assays

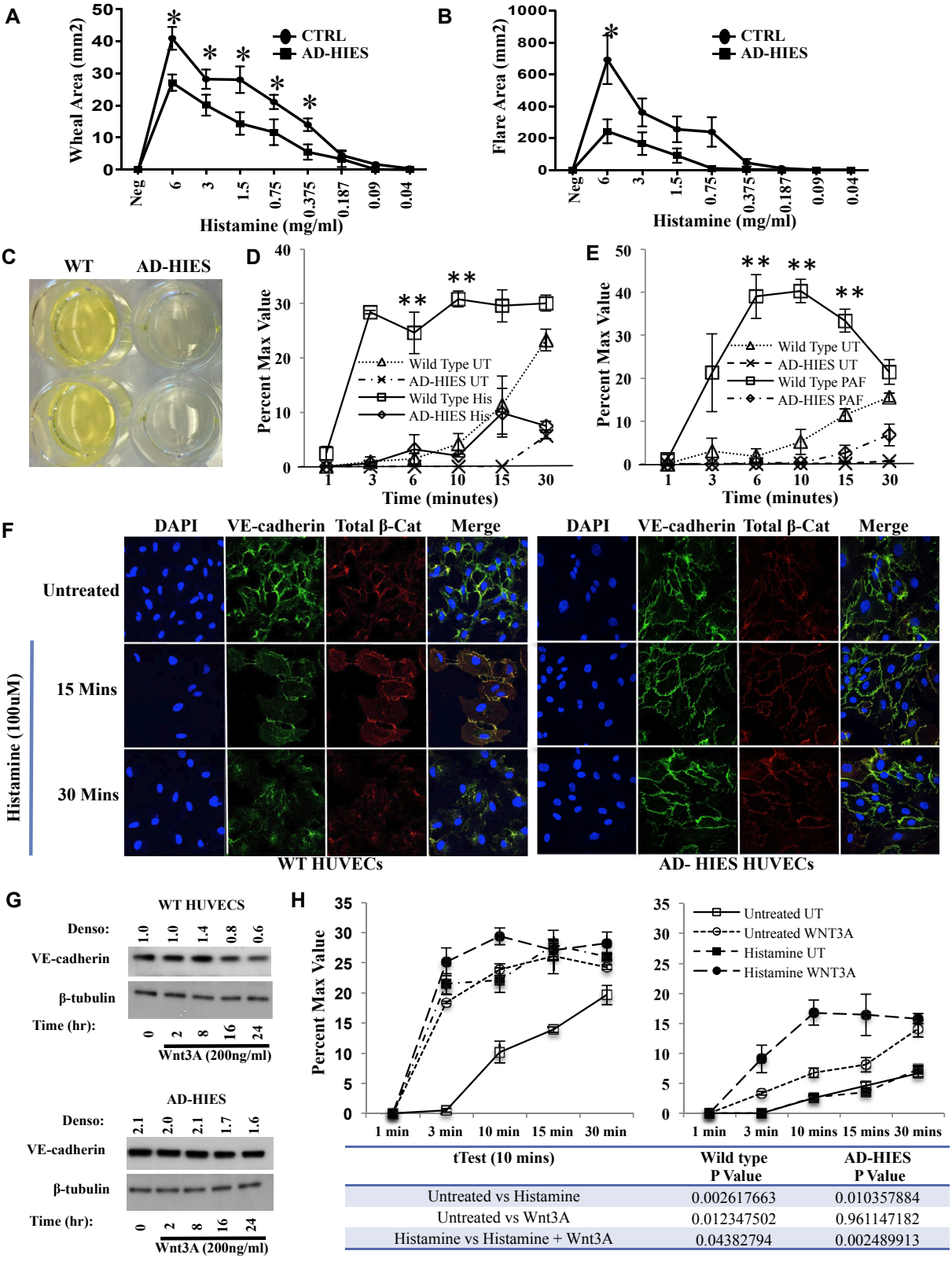
HUVECs were transfected with a 1-μg Cignal STAT3 Reporter (Qiagen, Hilden, Germany), as described above. Next, 24 hours after transfection, cells were treated with increasing amounts of the STAT3 agonist IL-11 for 20 hours and subjected to a Dual Luciferase reporter assay (Promega, Madison, Wis), according to the manufacturer's protocol.

For statistical analysis information, see the Methods section in this article's Online Repository.

RESULTS

Repeated injections with C188-9 reduce anaphylaxis severity in mice

To establish a model of systemic STAT3 inhibition, we pretreated WT mice with the STAT3 inhibitor C188-9.[34] Treatment for 5 to 7 days profoundly diminished the duration and severity of IgE-mediated anaphylaxis, whereas treatments for 1 or 4 days were ineffective or mildly effective, respectively



tTest (10 mins)	Wild type P Value	AD-HIES P Value
Untreated vs Histamine	0.002617663	0.010357884
Untreated vs Wnt3A	0.012347502	0.961147182
Histamine vs Histamine + Wnt3A	0.04382794	0.002489913

(Fig 1, *A*, and see Fig E1, *A* and *B*, in this article's Online Repository at www.jacionline.org). C188-9 injections were well tolerated and resulted in efficient reduction of STAT3 phosphorylation (serine 727, the residue phosphorylated by vascular mediators, such as histamine) in peritoneal cells (see Fig E1, *C*).

Degranulation in mouse mast cells, but not human mast cells, is STAT3 independent

Surprisingly, the reduced IgE-mediated anaphylactic responses in C188-9-treated mice did not correspond with decreases in early release (90 seconds) of the markers of mast cell activation histamine and MCPT-1 (Fig 1, *B*) or alterations in their subsequent metabolism (30 minutes; see Fig E1, *D*). Similarly, the reduced anaphylaxis observed in a *Stat3*-mutated mouse model of AD-HIES[9] did not correspond to significant differences in the release of histamine after challenge (see Fig E1, *E*). To resolve the discrepancy between the mouse and *in vitro* data showing STAT3 to be important for human mast cell degranulation,[9] we examined the effect of C188-9 inhibition on mouse BMMCs and PDMCs. Unlike human-derived mast cells, which showed a 30% to 40% reduction in degranulation when pretreated with C188-9 for 5 days (see Fig E2, *A* and *B*, in this article's Online Repository at www.jacionline.org), IgE/antigen-induced degranulation in BMMCs or PDMCs (see Fig E2, *C* and *D*) was unaltered by C188-9 treatment. Only when PDMCs were cultured in the presence of IL-6 did the degranulation of these cells become mildly sensitive to C188-9 treatment. Short-term treatment with C188-9 for 24 hours had no effect on degranulation in human mast cells (see Fig E2, *E* and *F*).

The effects of C188-9 in human mast cells required long-term treatment (5-7 days). This was not due to slow uptake or action of the inhibitor because incubation of LAD2 with C188-9 for just 1 hour effectively prevented STAT3 phosphorylation (Tyr-705) induced by IL-6 stimulation (see Fig E3, *A* and *B*, in this article's Online Repository at www.jacionline.org). The reduced phosphorylated STAT1 response under IL-6–stimulating conditions (see Fig E3, *A* and *B*) was likely due to lower levels of IL-6–induced STAT1/STAT3 heterodimers because no inhibition of STAT1 phosphorylation was seen after IFN-γ stimulation, a more pure STAT1 activator, in C188-9-treated LAD2 cells (see Fig E3, *C* and *D*).

These data suggest that the mechanism by which STAT3 inhibition prevents anaphylactic responses is not solely dependent on the effects on mast cell degranulation, and in the mouse mast cell degranulation is not as dependent on STAT3.

STAT3 inhibition reduces vascular permeability in response to mast cell mediators

To determine the compartment responsible for the impaired anaphylactic response in STAT3-inhibited conditions, we looked for differences in the responsiveness to mast cell mediators themselves, such as histamine and PAF. C188-9-treated or *Stat3*–mutated mice were protected against anaphylaxis induced by a bolus of 5 μmol of histamine (Fig 1, *C* and *D*). In addition, C188-9 treatment reduced the severity and duration of passive systemic anaphylaxis induced by the potent mediator PAF (Fig 1, *E*) and prevented death associated with PAF administration (Fig 1, *F*). Correlating with the protective effect of C188-9 in the anaphylactic response, the increase in hematocrit levels after treatment with either histamine or PAF (reflective of increases in vascular permeability) was completely blunted in mice treated with C188-9 (Fig 2, *A* and *B*), and extravasation of Evans Blue dye during a cutaneous hypersensitivity reaction induced by IgE/antigen (Fig 2, *C*) or compound 48/80 (Fig 2, *D*) was significantly reduced in mice treated with C188-9 compared with that seen in mice treated with vehicle alone.

We then examined whether resistance to anaphylaxis was afforded by reduced responsiveness of the vascular endothelium to mast cell mediators. Treatment of MLE cell cultures with C188-9 for 5 days resulted in a pronounced decrease in cell permeability after treatment with histamine (100 μmol/L; Fig 2, *E*) or PAF (10 μg/mL; Fig 2, *F*), indicating that STAT3 inhibition in mice protects against anaphylaxis independently of mast cell responsiveness by impeding vascular mediator–induced endothelial permeability.

STAT3 inhibition reduces permeability in HUVECs by stabilizing VE-cadherin/β-catenin junctions and prevents Src-mediated adherens junction dissolution

We next investigated the effect of STAT3 inhibition in WT HUVECs. After establishing an appropriate dose and confirming that the inhibitor led to very low STAT3 transcriptional activity (see Figs E4 and E5 in this article's Online Repository at www.jacionline.org), treatment with C188-9 for 5 days resulted in a significant decrease in WT HUVEC permeability *in vitro* after exposure to histamine (100 μmol/L) or PAF (10 μg/mL; Fig 3, *A* and *B*).

VE-cadherin and its intracellular anchor, β-catenin, are junctional proteins associated with endothelial cell adhesion. Because dissociation of β-catenin from VE-cadherin is required

FIG 4. HUVECs from patients with AD-HIES exhibit intrinsically decreased vascular permeability through increased VE-cadherin junctions. Patients with AD-HIES and healthy control subjects (n = 10 per group) underwent skin prick tests with increasing amounts of histamine. **A** and **B,** The area of the wheal (Fig 4, *A*) and flare (Fig 4, *B*) was recorded. **C,** Image of the bottom of a transwell chamber after exposure of WT HUVECs and HUVECs from patients with AD-HIES to the fluorescent reporter FITC-Dex (1 mg/well) for 30 minutes. **D** and **E,** *In vitro* permeability assay of WT HUVECs and HUVECs from patients with AD-HIES exposed to histamine (100 μmol/L; Fig 4, *D*) or PAF (10 μg/mL; Fig 4, *E*; n = 3 per group). *His,* Histamine; *UT,* untreated. Data are representative of 3 independent experiments. **F,** Immunofluorescent analysis of VE-cadherin (green), total β-catenin (red), and fluorescent isothiocyanate–dextran (DAPI; blue) in WT HUVECs and HUVECs from patients with AD-HIES after exposure to histamine (100 μmol/L). **G,** Western analysis of VE-cadherin in WT HUVECs and HUVECs from patients with AD-HIES after exposure to Wnt3A (200 ng/mL). Data are representative of 2 independent experiments. **H,** *In vitro* permeability assay of WT HUVECs (*left panel,* n = 3 per group) and HUVECs from patients with AD-HIES (*right panel,* n = 3 per group) pretreated with Wnt3A (200 ng/mL) and subsequently exposed to histamine. Data are representative of 3 independent experiments. Data are means ± SEMs. $*P < .05$ and $**P < .01$.

for increased vessel permeability, we next characterized VE-cadherin/β-catenin complexes after STAT3 inhibition. Immunofluorescent analysis confirmed colocalization of VE-cadherin and β-catenin in HUVECs (see Fig E5, *B-C*). As expected, histamine treatment resulted in junction dissolution of VE-cadherin and β-catenin in vehicle-treated WT HUVECs (Fig 3, *C*, left panel), with the reduction of β-catenin appearing to be a selective disruption of the active nonphosphorylated form (Fig 3, *D*, left panel). The dissolution was quantitated across multiple 6.5-μm sections of the cell membrane (see Fig E6 in this article's Online Repository at www.jacionline.org).

STAT3 inhibition with C188-9 prevented histamine-mediated dissociation of both VE-cadherin and β-catenin (Fig 3, *C*, right panel) and active β-catenin dissociation (Fig 3, *D*, right panel). This inability to dissociate was due to a failure of VE-cadherin to become phosphorylated in the presence of the STAT3 inhibitor. In DMSO-treated HUVECs histamine treatment resulted in phosphorylation of the histamine-specific residue of STAT3 (S727), but not STAT3 (Y705), and phosphorylation of VE-cadherin (needed for dissolution of the junction). In STAT3-inhibited (C188-9-treated) HUVECs very little activity was detected for phosphorylation of STAT3 or VE-cadherin, suggesting that the mechanism regulating VE-cadherin–associated junction dissolution is being constrained (Fig 3, *E*).

Histamine skin prick test responses are reduced among patients with AD-HIES

Previous data from patients with dominant negative STAT3 mutations have demonstrated preserved responses to a single and potentially saturating dose of histamine when examining only wheal diameter.[9] In light of diminished systemic responsiveness to histamine in the Stat3mut mouse, we performed skin prick tests with serial dilutions of histamine and measured the areas of the elicited wheal-and-flare response to more accurately measure potential differences in cutaneous responsiveness. We found that patients with AD-HIES demonstrated significantly diminished histamine-induced wheal (Fig 4, *A*) or flare (Fig 4, *B*) area at several dilutions.

Intrinsic increase in VE-cadherin/β-catenin stability and reduced permeability among HUVECs from patients with AD-HIES

Similar to C188-9-treated WT HUVECs, HUVECs derived from 2 different newborns with AD-HIES were less permeable than WT HUVECs when exposed to FITC-Dex alone for 30 minutes (Fig 4, *C*) or stimulated with histamine (100 μmol/L; Fig 4, *D*) or PAF (10 μg/mL; Fig 4, *E*) in the presence of FITC-Dex. HUVECs from patients with AD-HIES were also resistant to the dissolution of VE-cadherin/total β-catenin junctions and the active form of β-catenin after histamine treatment (Fig 4, *F*, and see Fig E7, *A* and *B*, in this article's Online Repository at www.jacionline.org). β-Catenin is a well-established downstream target of the canonical Wnt pathway. To determine the level at which the dissolution defect exists, we found that although Wnt3A RNA and protein levels were undetectable (data not shown), HUVECs from patients with AD-HIES treated with exogenous Wnt3A (200 ng/mL) led to decreased VE-cadherin and active β-catenin expression at 24 hours in WT HUVECs and HUVECs from patients with AD-HIES (Fig 4, *G*, and see Fig E7, *C*), partially restoring the permeability of HUVECs from patients with AD-HIES in response to histamine (Fig 4, *H*).

Reduced adherens junction dissolution is due to inhibited Src signaling

Because calcium signaling was normal after addition of histamine to STAT3-attenuated HUVECs (see Fig E8, *A* and *B*, in this article's Online Repository at www.jacionline.org; also seen in MLE cells in Fig E6, *C*) and the failure of the adherens junctions to dissociate could be rescued by exogenous Wnt3A, we hypothesized that other upstream signaling defects might exist in STAT3-inhibited or mutated HUVECs, as was seen in STAT3-inhibited mast cells.[9] Histamine receptor signaling through proximal signaling Src molecules is at least in part responsible for VE-cadherin responses.[35] In DMSO-treated HUVECs histamine treatment resulted in an increase in the phosphorylation of SrcY416 (activating Src), which was mirrored by phosphorylation of SrcY527 (inactivating Src). However, in C188-9-treated HUVECs very little Src phosphorylation was detected (Fig 5, *A*, right). Furthermore, at early time points, histamine-induced Src and VE-cadherin phosphorylation is delayed in HUVECs from patients with AD-HIES compared with WT HUVECs (Fig 5, *B*; pSrc(Y416), lane 2 vs lane 10; pVE-Cad, lane 2 vs lane 10). Concomitant increases in total expression of PTEN (Fig 5, *C*), a lipid and protein phosphatase known to inhibit Src,[36] and a reduction in the inactive and phosphorylated form of PTEN (Fig 5, *A*, left) in HUVECs from patients with AD-HIES were also observed, suggesting the failure to downregulate VE-cadherin might have been due to excessive PTEN-mediated inhibition of Src. Therefore a failure (C188-9) or delay (AD-HIES) in activation of Src signaling potentially caused by excess PTEN expression contributes to impaired permeability in these STAT3-inhibited or mutated cells.

Mutated STAT3 leads to decreased expression of the transcriptional target mir17-92, which regulates elements of the endothelial response to histamine

To demonstrate that direct phosphorylation of STAT3 was not responsible for the effects on vascular permeability, we treated HUVECs with IL-11, which acts on endothelial cell–specific IL-11 receptors, which induced STAT phosphorylation in WT HUVECs and HUVECs from patients with AD-HIES within 10 minutes. However, short-term IL-11 treatment (see Fig E9, *A*, in this article's Online Repository at www.jacionline.org) did not affect vascular permeability of WT HUVECs or HUVECs from patients with AD-HIES alone or in combination with histamine treatment (see Fig E9, *B-D*).

The microRNA cluster mir17-92 is upregulated by STAT3[25] and strongly inhibits PTEN, a phosphatase that regulates Src signaling, under normal conditions.[36] Therefore we found that the STAT3 agonist IL-11[37] induced mir17-92 expression in WT HUVECs within 3 hours and was sustained over 24 hours (Fig 5, *D*), although not in HUVECs from patients with AD-HIES. In agreement with reduced mir17-92 expression in STAT3-mutated cells, E2F1 and siah E3 ubiquitin protein ligase 1(SIAH1) (regulators of β-catenin signaling) expression was increased (Fig 5, *E*), which might contribute to the increased basal

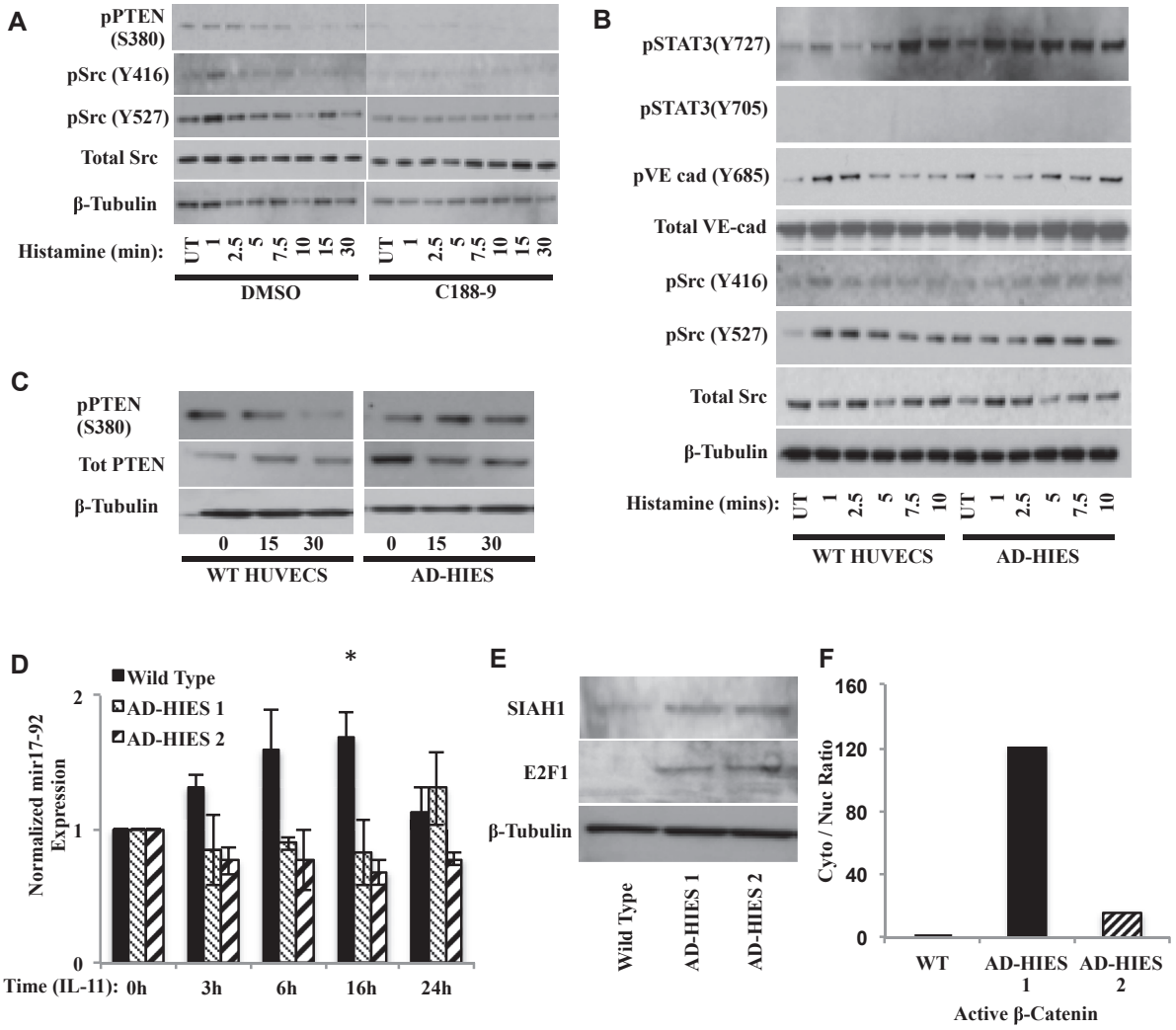


FIG 5. HUVEC permeability is regulated by STAT3-induced mir17-92 and can be restored in cells from patients with AD-HIES through the canonical Wnt signaling pathway. **A,** Western blot analysis of phosphorylated Src, total Src, and phosphorylated PTEN in DMSO- and C188-9-treated HUVECs after exposure to histamine (100 μmol/L). **B** and **C,** Western blot analysis of phosphorylated VE-cadherin, total VE-cadherin, active (nonphosphorylated) β-catenin, total β-catenin, phosphorylated Src, total Src, phosphorylated PTEN, total PTEN, and phosphorylated STAT3 in WT HUVECs and HUVECs from patients with AD-HIES after exposure to histamine (100 μmol/L). Data are means ± SEMs. *$P < .05$. **D,** Real-time PCR analysis of mir17-95 RNA expression in IL-11 (100 ng/mL)–treated WT HUVECs and HUVECs from patients with AD-HIES. Data are representative of 3 independent experiments. **E,** Western blot analysis of SIAH1 and E2F1 expression in WT cells and cells from patients with AD-HIES. Data are representative of 2 independent experiments. **F,** Analysis of cytoplasmic verses nuclear active β-catenin in WT HUVECs and HUVECs from patients with AD-HIES.

cytoplasmic to nuclear active β-catenin observed in HUVECs from patients with AD-HIES (Fig 5, *F*).

Because HUVECs from patients with AD-HIES have impaired Src signaling, mir17-92 cluster induction, and enhanced PTEN expression, we asked whether exogenous expression of mir17-92 or inhibition of PTEN could rescue the permeability defect. Transfection with a mir19a mimic led to a decrease in total PTEN expression in WT HUVECs (nearly 100%) and HUVECs from patients with AD-HIES (33%; Fig 6, *A*) and rescued the vascular permeability defect in HUVECs from patients with AD-HIES (see Fig E10 in this article's Online Repository at www.jacionline.org). After transfection, total PTEN levels were increased 1.21-fold in HUVECs from patients with AD-HIES compared with those in WT HUVECs. In addition, transfection of an mir19a antagomir in WT HUVECs under STAT3-stimulating conditions led to increased total PTEN levels (see Fig E10, *B*).

To demonstrate that STAT3 inhibition, as opposed to just STAT3 mutation, also increases total PTEN expression, we treated WT HUVECs with C188-9 for up to 7 days and found increased total PTEN levels (Fig 6, *B* and see Fig E10, *C*). Treatment with a PTEN inhibitor (VO-OHpic) increased Src activity (Fig 6, *C*) and rescued the permeability defect seen in C188-9-treated HUVECs (Fig 6, *D*) and HUVECs from patients

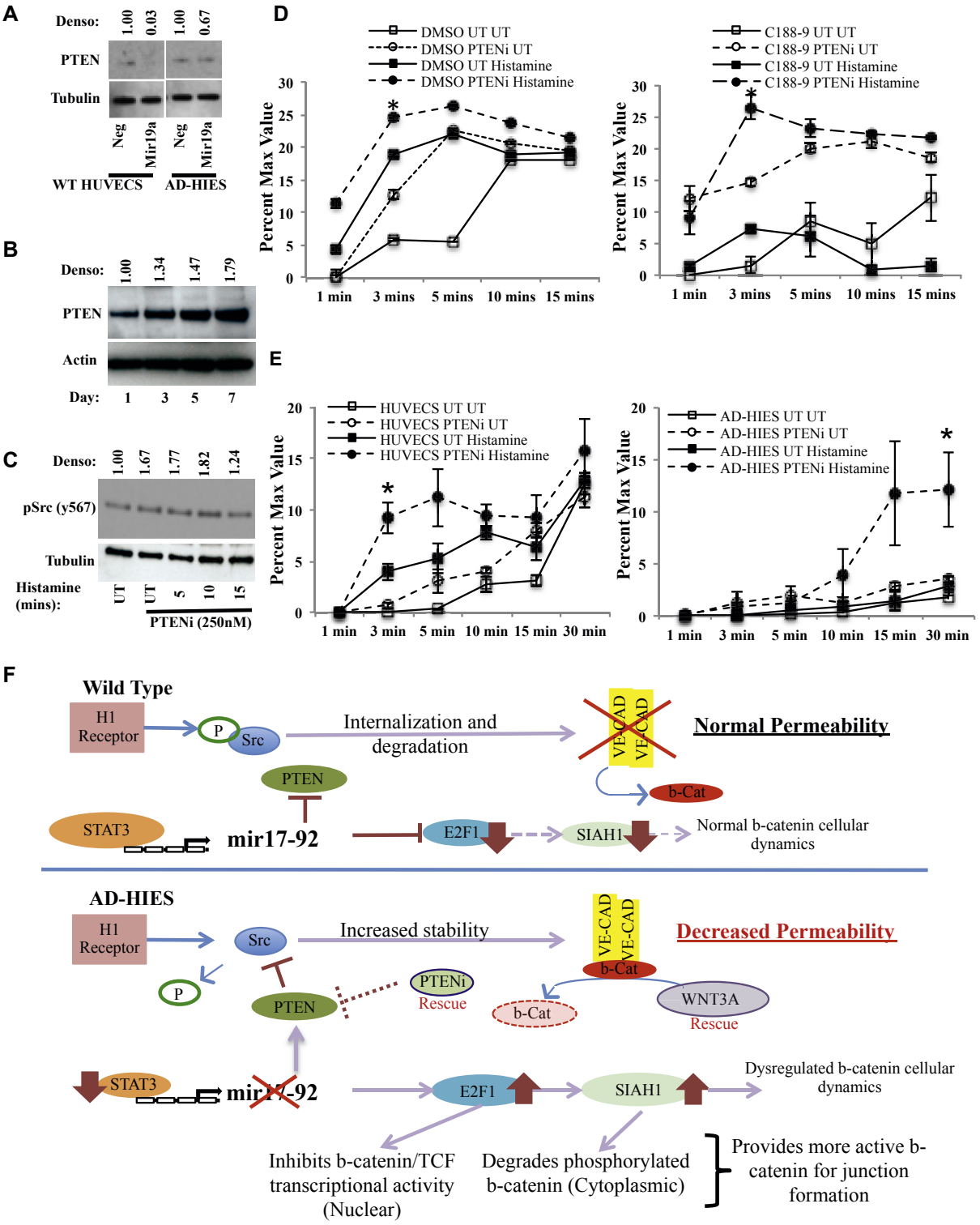


FIG 6. PTEN inhibition restores STAT3-inhibited or mutated HUVECs permeability. **A,** PTEN expression in WT HUVECs and HUVECs from patients with AD-HIES after transfection with a mir19 mimic. **B,** PTEN expression in STAT3-inhibited WT HUVECs over 7 days. **C,** Expression of phosphorylated Src in PTEN inhibitor–pretreated HUVECs (1 hour) that were subsequently exposed to histamine (100 μmol/L). **D** and **E,** *In vitro* permeability assay of DMSO- or C188-9-treated HUVECs (Fig 6, *D*) or WT HUVECs and HUVECs from patients with AD-HIES treated with a PTEN inhibitor (1 hour) and subsequently exposed to histamine (100 μmol/L; Fig 6, *E*). Data are means ± SEMs. **P* < .05, **F,** Model of STAT3 regulation of vascular permeability in WT HUVECs and HUVECs from patients with AD-HIES. In WT cells histamine treatment leads to Src phosphorylation, resulting in dissociation of β-catenin and internalization and degradation of VE-cadherin. Under conditions of normal STAT3 signaling, STAT3 induces expression of the mir17-92 microRNA cluster

with AD-HIES (Fig 6, *E*). Of note, in DMSO-treated or WT HUVECs, treatment with the PTEN inhibitor alone was sufficient to partially increase permeability (see Fig 6, *D* and *E*, left panels), correlating to the 67% increase seen in basal phosphorylated Src levels in the presence of the PTEN inhibitor alone (Fig 6, *C*). Taken together, these data demonstrate that STAT3 inhibition promotes PTEN upregulation, increased PTEN impairs Src signaling, and inhibition of PTEN through small molecule– or mir17-92–mediated inhibition can restore Src signaling and overcome the decreased vascular leakiness caused by impaired STAT3 signaling.

DISCUSSION

We describe a STAT3-dependent effect in human subjects and mice on adherens protein stability, which leads to altered vascular permeability and protection from systemic anaphylaxis, suggesting that certain clinical responses to mast cell mediators could be controlled through STAT3 inhibition.

In agreement with our previous finding that STAT3 mutant patients with AD-HIES are less susceptible to anaphylaxis compared with other highly atopic subjects,[9] IgE-mediated passive systemic anaphylaxis was more attenuated in mice that were treated with the STAT3 inhibitor C188-9.

Although mitochondrial STAT3 has been shown to play a role in IgE/antigen-mediated mast cell degranulation[10] in rodent models, in the present study STAT3 inhibition did not result in a detectable difference in circulating mast cell mediators (histamine and MCPT-1) in an *in vivo* anaphylaxis model or defective β-hexosaminidase release *in vitro* in a mast cell activation assay. These differences can be reconciled by the fact that Erlich et al[10] mainly used different concentrations of inhibitor and different cell lines that might have resulted in different on- and off-target effects. We found that hematocrit levels and local plasma transudate in response to IgE/antigen, but also to histamine alone, were significantly lower in C188-9-treated mice compared with those in untreated mice. These data suggest that STAT3 inhibition interferes with a common pathway regulating vascular permeability and were supported by a decrease in mediator-induced permeability in HUVECs *in vitro*. As such, although human mast cell function can be affected by reduced STAT3 signaling, there appears to be a significant effect on endothelial cell responsiveness to mast cell mediators that contributes to the protection from immediate hypersensitivity reactions. Of note, the proximal histamine-signaling defect observed here was similar to that seen through FcεRI in STAT3-deficient human mast cells and might suggest a similar underlying cause.[9]

The inflammatory cytokine IL-6 has also been shown to contribute to vascular leakage through IL-6–related trans-signaling by using STAT3 and extracellular signal-regulated kinase.[22] Although HUVECs do not express the IL-6 receptor,[38] IL-11–mediated phosphorylation of STAT3 in HUVECs was observed but had no effect on vascular permeability after short-term stimulation. Coupled with the fact that mice had to be pretreated with a STAT3 inhibitor for multiple days before any effect on permeability was observed, it is surmised that STAT3-linked transcriptionally controlled events were required to affect permeability.

The microRNA cluster mir17-92 is a direct downstream target of STAT3.[25] Upregulation of mir17-92 leads to direct suppression of PTEN and E2F1,[26] negative regulators of Src and β-catenin/transcription factor signaling, respectively. Mir17-92 expression leads to a concomitant reduction in E2F1-dependent expression of the E3-ubiquitin ligase SIAH1.[39] Our data demonstrate that this pathway is active in HUVECs that have intact STAT3 but suppressed in STAT3-mutated HUVECs from patients with AD-HIES. That suppression appears to be occurring through 2 routes: (1) increased PTEN preventing Src-related dissolution of VE-cadherin and (2) increased E2F1/SIAH1 altering β-catenin cellular dynamics, increasing adherens junction anchor availability. Of note, increased E2F1 expression leads to p53-dependant repression of vascular endothelial growth factor,[40,41] which normally destabilizes VE-cadherin junctions,[19] which might also contribute to this phenotype. This observation is supported by the fact that E2F1 knockout mice have increased vascular permeability, rapid PTEN reduction is seen during anaphylaxis, and PTEN-deficient mice have enhanced allergic responses and increased vascular permeability.[40,42-44] Although the link between STAT3, the miR17-92 complex, PTEN expression, and Src-mediated responsiveness to histamine appears intriguing, further work will be necessary to definitively describe this mechanism.

In addition to β-catenin being a major component of VE-cadherin junctions, it is also a well-established target of the canonical Wnt pathway.[45-47] Studies have shown that endothelial cell contact might be required to enhance colocalization of VE-cadherin and β-catenin at the cell membrane and that signaling through the canonical Wnt pathway (Wnt3A-mediated) disrupts the VE-cadherin/β-catenin interaction.[48] In addition, noncanonical Wnt signaling (Wnt5A) can repress Wnt/β-catenin signaling.[49,50] Consistent with these data, activation of the canonical Wnt signaling pathway in HUVECs decreased membrane-associated β-catenin levels and increased permeability, even in cells from patients with AD-HIES.

Because activation of the Wnt pathway or inhibition of PTEN can rescue permeability and normal calcium mobilization is seen in both WT HUVECs and HUVECs from patients with AD-HIES, it is likely that a focal pathway defect exists in patients with AD-HIES, as opposed to a global problem of vascular permeability. The model generated from our data is summarized in Fig 6, *F*.

This study shows that impaired STAT3 signaling reduces mast cell mediator–induced vascular permeability through enhanced

that suppresses PTEN, E2F1, and SIAH1 levels. This allows for normal regulation of Src signaling and normal distribution of β-catenin cellular dynamics and thus directly regulates the amount of VE-cadherin and β-catenin. In STAT3-mutated cells histamine treatment leads to no Src phosphorylation because of decreased expression of mir17-92 resulting in increased PTEN. This prevents the internalization and degradation of VE-cadherin, ultimately increasing its expression. Mir17-92 deficiency also allows for increased E2F1 and SIAH1 expression. This will degrade any phosphorylated β-catenin in the cytoplasm and inhibits nuclear translocation of nonphosphorylated cytoplasmic β-catenin, which provides more nonphosphorylated β-catenin to form adherens junctions, leading to decreased vascular permeability.

expression and stability of VE-cadherin/β-catenin complexes in vascular endothelial cells. Reduced STAT3 signaling protects against passive anaphylaxis in both mut-*Stat3* and WT mice treated with a *Stat3* inhibitor. Such inhibition might be useful in preventing severe immediate hypersensitivity reactions in a variety of settings. It will also be of great interest to learn in future studies how STAT3 inhibition can disrupt abnormal vascular permeability observed in other inflammatory and noninflammatory settings, in particular because inflammatory responses of patients with AD-HIES to infection are markedly curtailed.[51,52]

Clinical implications: Long-term functional ablation of STAT3 prevents vascular mediator–induced dissolution of adherens junctions and suggests that clinical conditions of excess vascular permeability of all sorts can be modulated through small-molecule STAT3 inhibition.

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