The TNF-like weak inducer of the apoptosis/fibroblast growth factor–inducible molecule 14 axis mediates histamine and platelet-activating factor–induced subcutaneous vascular leakage and anaphylactic shock

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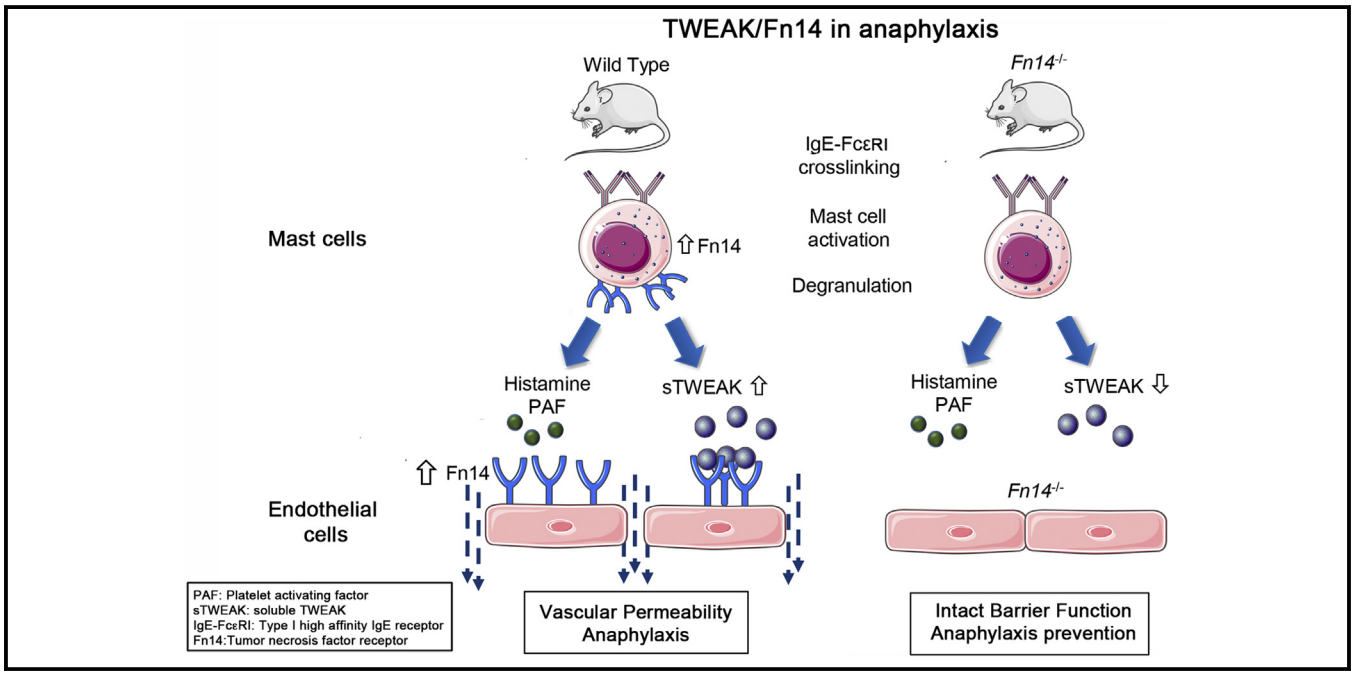
GRAPHICAL ABSTRACT



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Background: Anaphylaxis includes mast cell (MC) activation, but less is known about downstream mechanisms (ie, vascular permeability controlled by endothelial cells [ECs]). The TNF-like weak inducer of apoptosis (TWEAK) and its sole receptor, fibroblast growth factor–inducible molecule 14 (Fn14), belong to the TNF superfamily and are involved in proinflammatory responses.
Objective: We sought to investigate the role of TWEAK/Fn14 axis in anaphylaxis.
Methods: *In vivo* vascular permeability and mouse models of passive systemic anaphylaxis (PSA) and active systemic anaphylaxis were applied to wild-type (WT), *TWEAK*- and *Fn14*-deficient mice ($TWEAK^{-/-}$ and $Fn14^{-/-}$, respectively). Primary bone marrow–derived mast cells (BMMCs) and ECs from WT and $Fn14^{-/-}$ or $TWEAK^{-/-}$ mice were studied. The TWEAK/Fn14 axis was also investigated in human samples.
Results: Mice with PSA and active systemic anaphylaxis had increased Fn14 and TWEAK expression in lung tissues and increased serum soluble TWEAK concentrations. TWEAK and Fn14 deficiencies prevent PSA-related symptoms, resulting in resistance to decreased body temperature, less severe reactions, and maintained physical activity. Numbers of MCs after PSA are similar between genotypes in different tissue regions, such as ear skin and the trachea, tongue, peritoneum, lungs, and bone marrow. Moreover, *in vitro* studies revealed no differences in degranulation or mediator release between WT and $Fn14^{-/-}$ BMMCs after IgE-FcεRI stimulation. *In vivo* and *in vitro* histamine and platelet-activating factor administration increases Fn14 receptor expression in lungs and ECs. Moreover, Fn14 deficiency in ECs maintained *in vitro* impermeability when stimulated by mediators or activated BMMCs but not by $TWEAK^{-/-}$ BMMCs, indicating that Fn14 is crucial for endothelial barrier function. TWEAK/Fn14 deletion or TWEAK-blocking antibody prevented histamine/platelet-activating factor–induced vascular subcutaneous permeability. Circulating soluble TWEAK levels were increased in patients with anaphylaxis, and plasma from those patients increased Fn14 expression in ECs.
Conclusion: The TWEAK/Fn14 axis participates in anaphylactic reactions. Inhibition of TWEAK/Fn14 interaction could be efficacious in anaphylaxis therapy. (J Allergy Clin Immunol 2020;145:583-96.)

Key words: *Anaphylaxis, TNF-like weak inducer of apoptosis/fibroblast growth factor–inducible molecule 14 axis, vascular permeability, mast cells, endothelial cells*

Abbreviations used
ASA: Active systemic anaphylaxis
BBB: Blood-brain barrier
BMMC: Bone marrow–derived mast cell
DMEM: Dulbecco modified Eagle medium
DNP: Dinitrophenyl
EC: Endothelial cell
FITC: Fluorescein isothiocyanate
Fn14: Fibroblast growth factor–inducible molecule 14
HRP: Horseradish peroxidase
IIS-FJD: IIS-Fundación Jiménez Díaz
MAEC: Mouse aortic endothelial cell
MC: Mast cell
MLEC: Mouse lung endothelial cell
NO: Nitric oxide
PAF: Platelet-activating factor
PSA: Passive systemic anaphylaxis
sTWEAK: Soluble TWEAK
TW: Transwell 24-well cell culture insert
TWEAK: TNF-like weak inducer of apoptosis
WT: Wild-type

With rapid onset and potentially lethal reactions, anaphylaxis is the most aggressive manifestation of allergic disorders. Anaphylaxis is triggered by hypersensitivity reactions mediated by FcεRI-bound IgE antibodies in response to allergens, which in turn activate mast cells (MCs) and basophils. These immune cells are considered the main effectors and amplifiers of the allergic response, inducing release of mediators and triggering reactions in surrounding tissues.[1-3] Tryptase, histamine, and platelet-activating factor (PAF) are the main biochemical mediators in human anaphylaxis, although numerous studies using mouse models have proposed other mediators as playing a fundamental role.[4-6]

The action of released mediators produces changes in homeostasis, pulmonary vasodilation, vascular permeability, and altered contractile function in the main organs involved in anaphylaxis, causing respiratory and cardiovascular anomalies that can lead to life-threating episodes.[7,8] One of the most relevant downstream immune effects observed is endothelial layer breakdown. Growing evidence points to the endothelium as a key player in anaphylactic reactions because this active layer is highly involved in maintaining the vascular barrier and controlling homeostatic stability.[9,10]

TNF-like weak inducer of apoptosis (TWEAK) and its sole functional receptor, fibroblast growth factor–inducible molecule 14 (Fn14), are 2 members of the TNF ligand superfamily. Fn14 expression is low or absent in healthy tissues but highly upregulated after injury. However, TWEAK is constitutively expressed in several tissues and can be proteolytically processed by furin, leading to release of soluble TWEAK (sTWEAK).[11]

It has been demonstrated that the TWEAK/Fn14 axis is implicated in several cell functions, such as proliferation, migration, angiogenesis, apoptosis, and inflammation.[11-14] Current evidence supports the beneficial or deleterious roles of the TWEAK/Fn14 axis depending on disease stage or severity, making this axis a potential target for the development of novel therapies.[15,16] TWEAK/Fn14 interaction plays a crucial role in a variety of pathologic inflammatory disorders, such as atherosclerosis, cardiac dysfunction, diabetes, and chronic kidney disease.[17-20] TWEAK/Fn14 has also been suggested as a potential target to limit edema formation, a major problem in patients with stroke.[21] In addition, it has been suggested that TWEAK/Fn14 signaling is relevant to angiogenesis.[12,22] Recently, TWEAK expression in sputum has been correlated with noneosinophilic childhood asthma.[23] Our aim was to expand the existing knowledge of the biological functions of TWEAK/Fn14 in allergy and the pathogenesis of anaphylaxis, and in doing so, we discovered TWEAK to be a potential target of anaphylaxis because of findings that show Fn14 as a mediator of histamine- and PAF-induced vascular leakage.

METHODS

An expanded Methods section is available in this article's Online Repository at www.jacionline.org.

Animal experimental designs

Animal procedures were carried out in accordance with European Union Directive 2010/63/EU for the care and experimental use of animals. The protocols used, which bear the reference code PROEX: 391/15, received prior approval from the IIS-Fundación Jiménez Díaz (IIS-FJD) Ethics Committee and the competent authorities in the region of Madrid. *TWEAK*-deficient ($TWEAK^{-/-}$) and *Fn14*-deficient ($Fn14^{-/-}$) mice and their wild-type (WT) counterparts were generously provided by Biogen Inc (Cambridge, Mass); backcrossing of these animals onto the C57BL/6 strain has been reported previously.[24] Mouse models of systemic vascular permeability and both passive systemic anaphylaxis (PSA) and active systemic anaphylaxis (ASA) were previously described, and sham (control) groups were included in the different experimental models.[25] Anti–dinitrophenyl (DNP) IgE-sensitized mice challenged with PBS were included as controls for PSA. Similarly, a group of mice treated with pertussis toxin diluted in PBS and challenged with bovine serum albumine (BSA) was included as a control for ASA.

Mice were intravenously administrated drugs, and animals were killed at 1 hour to evaluate the effect of *in vivo* histamine (10 mg/kg) and PAF (2 μg/g). PAF induced severe hypothermia and resulted in spontaneous death of mice within 1 hour.[25,26] PSA-Miles assay mice were challenged with DNP together with 1% Evans blue dye administered intravenously (100 μL). Once mice were intravenously challenged, they were and observed, and pictures were taken 15 minutes later.

Histologic analysis

Perfusion fixation of the organs was carried out with 4% paraformaldehyde, and 4-μm paraffin cross-sections were stained with toluidine blue, naphthol AS-D chloroacetate esterase (Sigma-Aldrich, St Louis, Mo), hematoxylin and eosin, or Masson trichrome or were used for immunohistochemistry (anti-TWEAK, anti-Fn14 or anti-CD31), according to standard protocols. Specificity was determined by substituting the primary antibody with unrelated IgG. Expression of Fn14 and TWEAK was quantified in 3 fields per lung chosen at random and was represented as area of positive staining per square millimeter. Positive MCs were counted in 3 different sections per organ. Results were expressed as positive staining per square millimeter or number of cells counted per area of each tissue.

Clinical score

Two independent viewers carried out an observational study of symptoms indicating systemic anaphylaxis, and these were scored as follows: 0, no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes and mouth, diarrhea, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate; 3, labored breathing and cyanosis around the mouth and tail; 4, no activity after prodding or tremor and convulsion; and 5, death.[27,28]

Spontaneous activity after anaphylaxis

A computerized actimeter (Panlab/Harvard, Holliston, Mass) was used to study spontaneous motor activity in mice with PSA. Rectal temperatures measured with a traceable digital thermometer (model VWR I620-2000) were taken throughout the period of evaluation.

Flow cytometric analysis

Half a lung from each mouse was cut with sterile scalpels to a pâté consistency and incubated with 0.1% collagenase P for 30 minutes. The mix was passed across a 20-gauge syringe (4 times) and a cell strainer. Peritoneal cells were obtained, as previously described,[29] and bone marrow was obtained from the femur. Cell suspensions were analyzed by using flow cytometry after staining with specific and control isotypes antibodies: CD45 (R&D Systems, Minneapolis, Minn), FcεRIα (MAR-1)–fluorescein isothiocyanate (FITC; Invitrogen, Carlsbad, Calif), CD117 (c-Kit) allophycocyanin/Cy7 (BioLegend, San Diego, Calif), rat IgG2bκ isotype control allophycocyanin/Cy7, and Armenian hamster IgG isotype control FITC.

In vitro mouse endothelial cell cultures

Mouse aortic endothelial cells (MAECs) and mouse lung endothelial cells (MLECs) were isolated from WT or $Fn14^{-/-}$ aortas, as previously described, with slight modifications.[25]

In vitro vascular permeability assays

Endothelial barrier integrity was evaluated by using Transwell 24-well cell culture inserts (TWs), as previously described.[25]

Western blot analysis and immunofluorescence

Cell lysates of bone marrow–derived mast cells (BMMCs) were obtained by using tissue lysis buffer, separated under reducing conditions on SDS-PAGE gels, and transferred to polyvinylidene difluoride membranes. Proteins were detected with anti-Fn14 antibody, anti-tubulin primary antibodies, and horseradish peroxidase (HRP)–conjugated secondary antibody. Proteins were visualized with enhanced chemiluminescence.

Endothelial cells (ECs) were grown on gelatin-coated coverslips, allowed to reach confluence, stimulated with histamine and PAF, fixed, permeabilized, and stained with anti–β-catenin Alexa Fluor 488, Texas Red-X phalloidin, and nuclei were counterstained with Hoechst stain. Images were acquired with an inverted confocal microscope (LSM700; Carl Zeiss, Oberkochen, Germany). Confocal microscopic images shown are maximal projections of a z-series.

ELISA

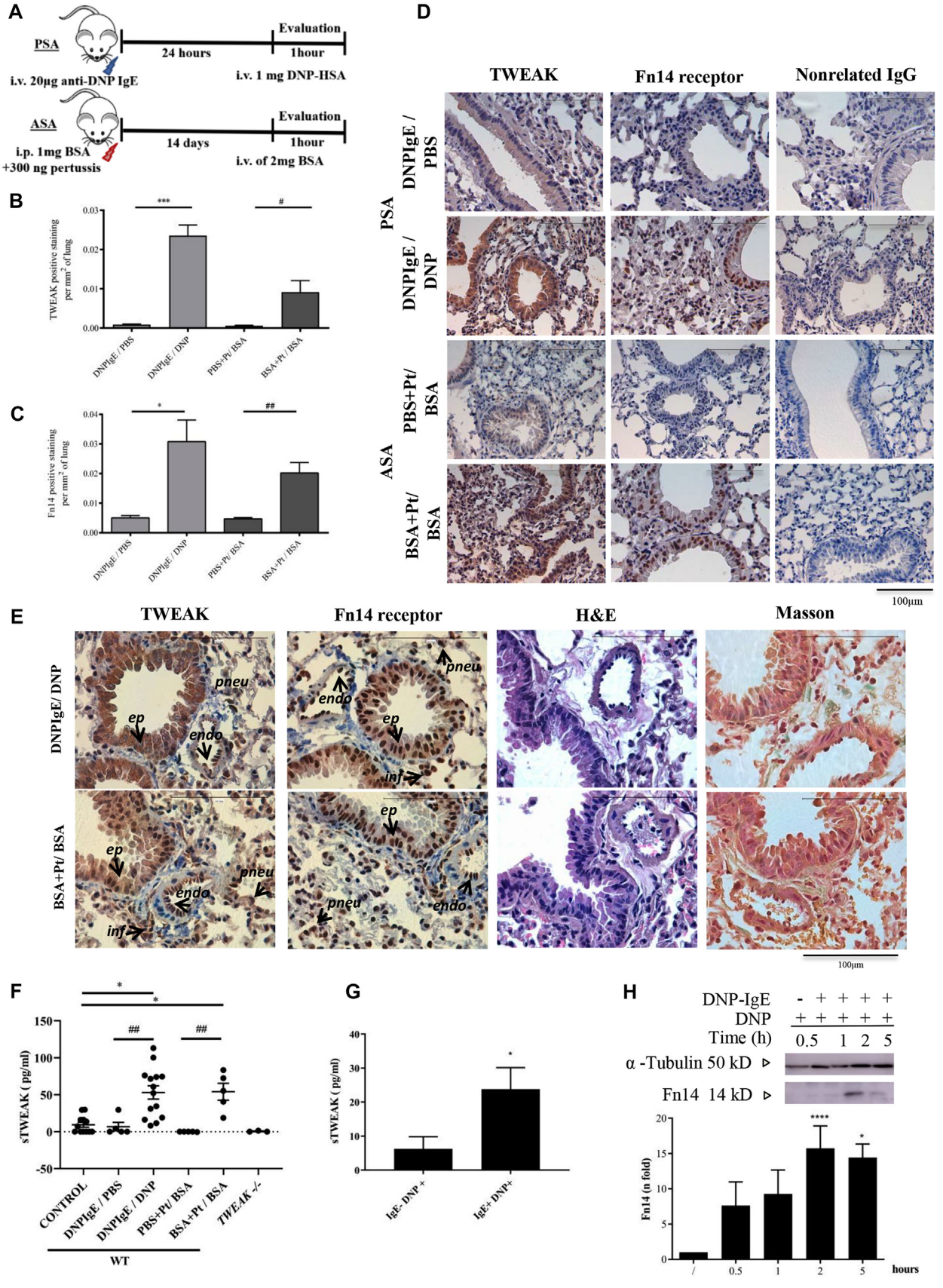
ELISAs were used according to the manufacturer's instructions. Human and mouse TWEAK and mouse MCPT-1 (mMCP-1) ELISAs were from Tebubio (Le Perry-en-Yvelines, France). IL-6 and TNF-α BMMC levels were analyzed by means of ELISA (R&D Systems). Murine IL-6 and TNF-α ELISA Kits from Diaclone (Besancon, France) were used to measure serum levels. An ELISA kit from Clod-Clone (Katy, Tex) was used to measure PAF levels. Nitric oxide (NO) serum levels (nitrite plus nitrate) were analyzed by using a colorimetric detection kit from Arbor Assays (Ann Arbor, Mich). Soluble IgE was measured by means of direct serum coating on the plate and detection with anti-IgE–HRP (Sigma-Aldrich), followed by TMB reagent (Thermo Fisher, Waltham, Mass).

Patients

The population studied included plasma samples from 55 patients with anaphylaxis and 24 healthy control donors. Subjects were included after providing informed consent, and the study was approved by the IIS-FJD ethics committee.

Statistical analysis

All values are expressed as means ± SEMs (numbers are noted in figure legends). Differences were evaluated with the GraphPad Prism 7.3 program (GraphPad Software, La Jolla, Calif) by using 1-way ANOVA, followed by the Bonferroni *post hoc* test (experiments ≥ 3 groups). The Mann-Whitney or Student *t* test was applied in experiments with 2 groups. Statistical significance was set at a P value of less than .05.


A
PSA
24 hours
Evaluation
1hour
i.v. 20µg anti-DNP IgE
i.v. 1 mg DNP-HSA
ASA
14 days
Evaluation
1hour
i.p. 1mg BSA
+300 ng pertussis
i.v. of 2mg BSA
B
TWEAK positive staining per mm² of lung
DNPIgE / PBS
DNPIgE / DNP
PBS+Pt/ BSA
BSA+Pt / BSA
C
Fn14 positive staining per mm² of lung
D
TWEAK
Fn14 receptor
Nonrelated IgG
PSA
ASA
DNPIgE / PBS
DNPIgE / DNP
PBS+Pt/ BSA
BSA+Pt/ BSA
100µm
E
TWEAK
Fn14 receptor
H&E
Masson
DNPIgE/ DNP
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CONTROL
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PBS+Pt/ BSA
BSA+Pt / BSA
TWEAK -/-
WT
G
sTWEAK (pg/ml)
IgE- DNP +
IgE+ DNP+
H
DNP-IgE
DNP
Time (h)
α -Tubulin 50 kD
Fn14 14 kD
Fn14 (n fold)
hours

RESULTS

Expression of TWEAK and the Fn14 receptor increase in mouse models of anaphylaxis

It has been demonstrated that sTWEAK levels are associated with the presence and progression of acute or chronic disease.[19,21,30,31] However, the plausible regulation of sTWEAK levels and Fn14 expression during anaphylaxis is still unknown. By using 2 experimental mouse models that simulate different degrees of allergic sensitivity, we have evaluated circulating sTWEAK concentrations in blood samples and TWEAK expression in lungs. Our experimental approach to PSA was based on systemic anti-DNP IgE sensitization over 24 hours, and ASA was induced by BSA, followed by challenge 14 days later (Fig 1, *A*). Both were compared with control (sham) mice and their respective sensitized control mice (anti-DNP IgE/PBS and PBS plus pertussis toxin/BSA). Interestingly, the histologic assessment and specific immunohistochemistry of TWEAK and Fn14 receptor in lung tissues showed marked increases in expression of both proteins when compared with the pulmonary tissue of untreated or sensitized mice (Fig 1, *B-D*). Increased TWEAK/Fn14 expression was evident in the respiratory epithelium of the bronchi and alveolar structures, mainly pneumocytes, inflammatory cells, and ECs (Fig 1, *E*). Additionally, an increase in sTWEAK level was observed in the sera of challenged mice from both models (Fig 1, *F*). Therefore *in vivo* the TWEAK/Fn14 axis was not upregulated by sensitization, whereas its increased expression was triggered in the setting of anaphylaxis.

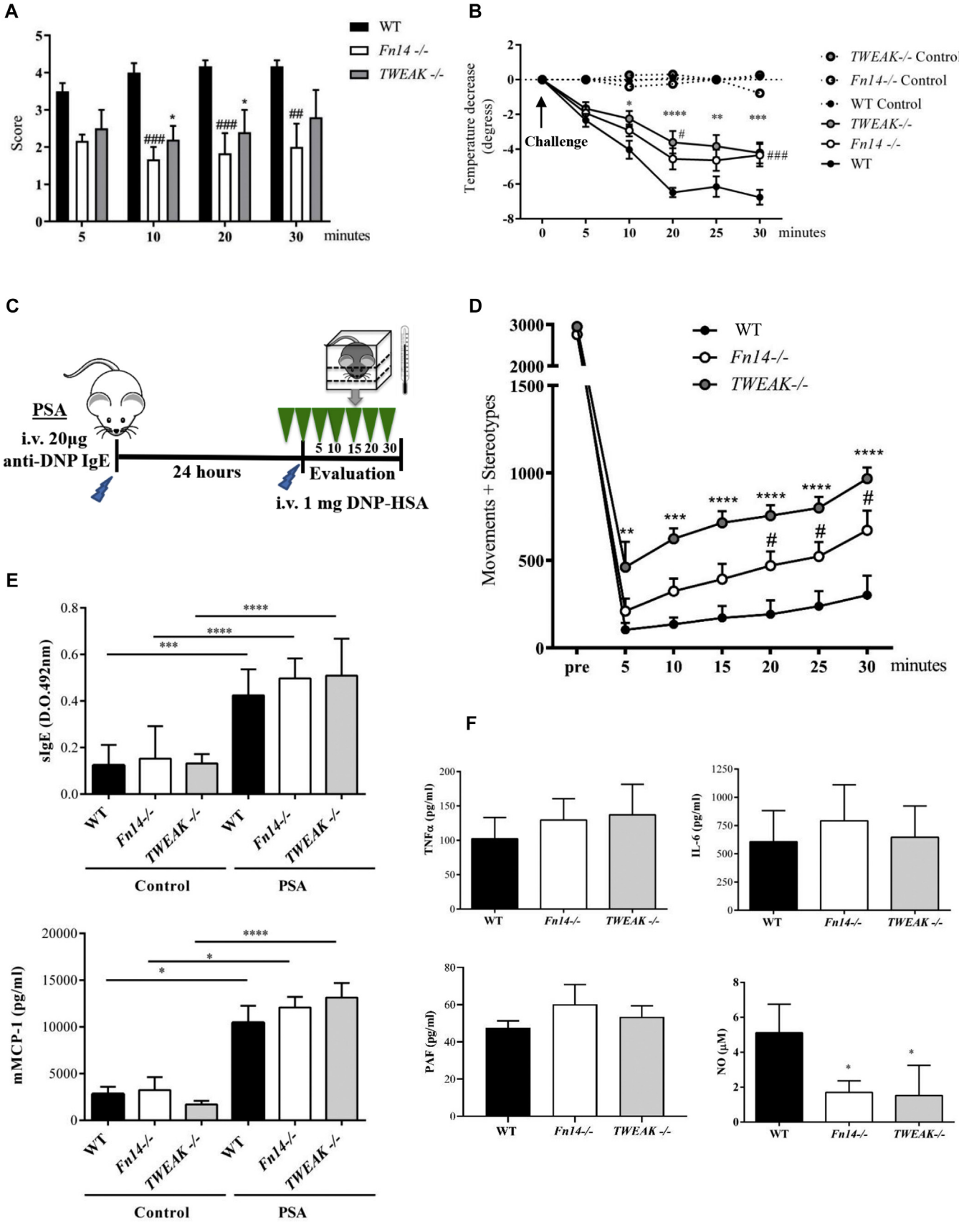
We next explored whether MCs could participate by secreting TWEAK, expressing the Fn14 receptor, or both in anaphylactic reactions. BMMCs were sensitized with anti-DNP IgE for 24 hours, followed by DNP challenge. We found that 1 hour after activation, BMMCs secreted a significant level of sTWEAK protein (Fig 1, *G*). Moreover, a marked increase in the level of Fn14 receptor was observed in BMMCs, which reach maximum expression after 2 hours (Fig 1, *H*). Together, *in vitro* results show that FcεRI aggregation induces sTWEAK release and Fn14 synthesis in BMMCs.

Symptoms of PSA are attenuated in *TWEAK*$^{-/-}$ and *Fn14*$^{-/-}$ mice

Modulation of TWEAK and Fn14 after induction of an allergic reaction suggested that this molecular axis participates in *in vivo* anaphylaxis. As a result, an observational study based on systemic symptoms of anaphylaxis was performed in *TWEAK*$^{-/-}$ and *Fn14*$^{-/-}$ mice. Scoring reflected clinical symptom severity observed in human subjects. Both *TWEAK*$^{-/-}$ and *Fn14*$^{-/-}$ mice with PSA had lower anaphylaxis symptom scores than the WT mice (Fig 2, *A*), suggesting the crucial role for TWEAK and Fn14 in the setting of anaphylaxis. Furthermore, body temperature and motor activity were also measured, and although hypothermia levels were observed in all genotypes, *TWEAK*$^{-/-}$ and *Fn14*$^{-/-}$ mice with PSA presented a significant resistance to the temperature decrease of almost 3 degrees compared with WT mice (Fig 2, *B*). However, any significant difference was observed in body temperature between either control or sensitized but not challenged WT, *TWEAK*$^{-/-}$, and *Fn14*$^{-/-}$ mice (see Fig E1 in this article's Online Repository at www.jacionline.org). Moreover, immunohistochemistry of TWEAK and Fn14 receptor expression performed in sensitized and sensitized/challenged *Fn14*$^{-/-}$ and *TWEAK*$^{-/-}$ mice, respectively, showed a similar pattern of expression of each molecule, indicating activation of the axis in both knockout mice after challenge (see Fig E2 in this article's Online Repository at www.jacionline.org).

Additionally, motor activity, as measured based on stereotyped and total movements, was monitored in a computerized actimeter chamber (Fig 2, *C*). Before challenge, all animals exhibited progressive and continuous wandering, with a high tendency toward stereotyped movements. After 5 to 15 minutes of recording baseline values, challenge was induced, triggering a marked initial decrease in motor and stereotyped activities, followed by a slight increase over the next approximately 30 minutes. As Fig 2, *D*, shows, *TWEAK*$^{-/-}$ and *Fn14*$^{-/-}$ mice had more cumulative movements than WT mice. Substantial differences in cumulative movements were seen between *TWEAK*$^{-/-}$ and WT mice, both in the initial stages and over the entire study period. Although in the case of *Fn14*$^{-/-}$ mice we did not observe significant motility differences with respect to WT mice during the first 15 minutes, subsequently, *Fn14*$^{-/-}$ mice were more active than WT mice.

FIG 1. Levels of sTWEAK and the Fn14 receptor increase in sera and lung tissue from mouse models of anaphylaxis and BMMCs. **A,** Schematic diagram showing PSA and ASA models. *i.p.*, Intraperitoneal; *i.v.*, intravenous. **B,** Quantification of TWEAK protein staining per square millimeter of lung 1 hour after challenge. DNPIgE/PBS, 4, DNPIgE/DNP, 9, PBS+Pt/BSA, 4; and BSA+Pt/BSA, 5. Student *t* tests were performed versus their respective controls for PSA (***P = .0002) and ASA (#P = .0370). **C,** Quantification of Fn14 protein staining per square millimeter of lung 1 hour after challenge. DNPIgE/PBS, 6; DNPIgE/DNP, 9; PBS+Pt/BSA, 6; and BSA+Pt/BSA, 5. Student *t* tests was performed versus their respective controls for PSA (*P = .0142) and ASA (##P = .0062). **D** and **E,** Representative images of anti-TWEAK, anti-Fn14 receptor, nonrelated IgG, hematoxylin and eosin *(H&E)*, and Masson staining of lung cross-sections of control mice, mice with PSA, mice with ASA, and their respective control mice. *Arrows* indicate positive pneumocytes *(pneu)*, epithelial cells *(ep)*, endothelial cells *(endo)*, and inflammatory cells *(inf)*. *Scale bars* = 100 μm. **F,** Graph shows sTWEAK levels measured by means of ELISA in WT mouse blood samples (pg/mL): Control, 12; DNPIgE/PBS, 5; DNPIgE/DNP, 14; PBS+Pt/BSA, 5; and BSA+Pt/BSA, 5. *TWEAK*$^{-/-}$ mice were used as controls. One-way ANOVA followed by the Bonferroni test was performed for mice with PSA and mice with ASA versus control mice (***P = .0002 and **P = .0082) and versus their respective control mice (PSA: ##P = .0044 and ASA: ##P = .0067). **G,** sTWEAK levels in BMMC supernatants after an hour of activation. Unpaired *t* test versus unstimulated cells in each genotype: *P = .0282 (n = 9); P = .6262 (not significant; n = 6). **H,** BMMCs were sensitized and stimulated with DNP-BSA for 15, 30, 60, or 120 minutes. Representative immunoblot for Fn14 receptor. Quantifications were normalized to α-tubulin. *Fn14*$^{-/-}$ BMMCs were used as controls. Data represent means ± SEMs for 5 to 7 experiments performed per cellular genotype. One-way ANOVA followed by the Bonferroni multiple comparisons test versus unstimulated WT BMMCs: *P = .0291 and ****P < .0001. *Pt,* Pertussis toxin.


A
WT
Fn14 -/-
TWEAK -/-
Score
minutes
B
TWEAK-/- Control
Fn14-/- Control
WT Control
TWEAK-/-
Fn14 -/-
WT
Temperature decrease (degress)
Challenge
minutes
C
PSA
i.v. 20µg anti-DNP IgE
24 hours
5 10 15 20 30
Evaluation
i.v. 1 mg DNP-HSA
D
WT
Fn14-/-
TWEAK-/-
Movements + Stereotypes
pre
minutes
E
sIgE (D.O.492nm)
mMCP-1 (pg/ml)
Control
PSA
F
TNFα (pg/ml)
IL-6 (pg/ml)
PAF (pg/ml)
NO (µM)

Next, soluble levels of soluble IgE and mMCP-1 were measured in serum samples of mice with PSA (Fig 2, *E*). Moreover, levels of other classical mediators released in anaphylactic reactions, such as TNF-α, IL-6, and PAF, were also determined, showing no significant differences between genotypes. However, we observed reduced serum NO production in *TWEAK*$^{-/-}$ and *Fn14*$^{-/-}$ mice compared with that in WT mice, showing the importance of NO in mouse models of anaphylaxis (Fig 2, *F*).[32]

A weakened anaphylactic response could be the result of reduced numbers of MCs.[6] Therefore comprehensive evaluation of morphology, localization, and quantification was performed in ear skin and the tracheas, tongues, and lungs of *TWEAK*$^{-/-}$ and *Fn14*$^{-/-}$ mice, and results were compared with those in WT mice with PSA. Toluidine blue and chloroacetate esterase staining showed a similar distribution pattern of degranulating MCs in tissues without a differences in cell numbers (Fig 3, *A* and *B*). Moreover, specific FcεRIα-CD117 cell staining was performed in lung tissue, peritoneal lavage fluid, and bone marrow cells of mice with PSA. Flow cytometric analysis showed no significant differences in the CD45^{+}FcεRIα^{+}CD117^{+} cell population either in the lung, peritoneum, and bone marrow between genotypes (Fig 3, *C*, and see Fig E3 in this article's Online Repository at www.jacionline.org).

In patients with anaphylaxis, IgE cross-linking activates MCs, which releases various mediators that contribute locally or systemically to exacerbate allergic reactions.[6] Mouse-derived BMMCs were sensitized with 100 ng/mL IgE anti-DNP and challenged with DNP-BSA to examine the role of the Fn14 receptor in MC degranulation and mediator release. Measurement of β-hexosaminidase showed a similar release profile from WT and *Fn14*$^{-/-}$ BMMCs (Fig 4, *A*). Additionally, detection of histamine, TNF-α, and IL-6 after 1 or 2 hours of stimulation also revealed a comparable pattern between WT and *Fn14*$^{-/-}$ BMMCs (Fig 4, *B*). Surprisingly, our *in vitro* data suggest that the role of the TWEAK/Fn14 axis in the setting of anaphylaxis is not linked to altered MC activation because Fn14 does not affect IgE-mediated degranulation or mediator release.

Deficiency of Fn14 prevents histamine- and PAF-induced vascular permeability in ECs

During anaphylaxis, homeostasis and vascular permeability are disturbed,[33] and a number of studies have demonstrated that 2 of the main mediators released in anaphylaxis, histamine and PAF, are the primary mediators of these processes.[34,35] It is also known that Fn14 expression is modified by TWEAK in different cell types[36] and that TWEAK can promote rupture of the blood-brain barrier (BBB).[37] Therefore we speculated as to whether the TWEAK/Fn14 pathway might play a role, participating in barrier disruption induced by histamine and PAF. Primary MAECs incubated with those mediators showed upregulation of Fn14 expression (Fig 5, *A*). In addition, the increased expression of Fn14 receptor was observed in lungs of histamine- and PAF-treated mice (Fig 5, *B*). Specific expression of Fn14 receptor in ECs was demonstrated by means of CD31 staining in consecutive lung sections of mice with PSA and ASA (Fig 5, *C*, and see Fig E4 in this article's Online Repository at www.jacionline.org).

Next, we examined whether a lack of Fn14 had an effect on histamine/PAF-inducing morphologic changes on WT and *Fn14*$^{-/-}$ endothelial monolayers. After stimulation, intracellular changes are associated with cytoskeletal reorganization and loss of adherent junctions, causing cell contraction and endothelial permeability.[38] Thus, after addition of either histamine or PAF for 15 minutes, confluent MAEC monolayers were fixed and stained for β-catenin and F-actin. These active mediators increased stress fiber disruption (visualized with Texas Red-X phalloidin staining) and rupture of the junctions (β-catenin) in WT MAECs. However, β-catenin–lined structure and F-actin organization were clearly shown in *Fn14*$^{-/-}$ MAECs, which exhibited a distinct junction assembly associated with cortical actin (Fig 5, *D*).

To determine whether those differences in stress fiber and junction pattern observed between WT and *Fn14*$^{-/-}$ MAECs could have an effect on vascular permeability, we functionally characterized the effect of histamine and PAF on barrier dysfunction in those cells. A significant increase in leakage was observed in response to both mediators on WT MAEC monolayers. However, *Fn14*$^{-/-}$ MAECs completely lacked mediator-induced vascular permeability (Fig 5, *E*). To extend these results, experiments were performed in parallel with MLECs, revealing a similar effect (Fig 5, *F*).

As the cellular microenvironment has become a focal point of research efforts, functional approaches evaluating the direct effect of activated BMMCs on endothelial monolayers have compared the interplay between cell types and genotypes. In our study IgE-sensitized and DNP-stimulated WT, *Fn14*$^{-/-}$, and *TWEAK*$^{-/-}$ BMMCs were cocultured with WT and/or *Fn14*$^{-/-}$ endothelial monolayers for 1 hour, simulating a microenvironment using co-cellular TW experiments. Both WT and *Fn14*$^{-/-}$ BMMCs-

FIG 2. TWEAK/Fn14 genetic deletion decreases PSA. Experimental mouse models were addressed in WT, *TWEAK*$^{-/-}$, and *Fn14*$^{-/-}$ mice. Animals were sensitized with anti-DNP IgE before challenge with DNP–human serum albumin. **A,** Score indicating symptom severity was applied. Two-way ANOVA followed by Bonferroni test: WT vs *Fn14*$^{-/-}$ mice, ###*P* = .0006, ##*P* = .0014; WT vs *TWEAK*$^{-/-}$ mice, **P* < .02 (n = 6 of each genotype). **B,** Temperature decrease. Two-way ANOVA followed by the Bonferroni test: WT vs *Fn14*$^{-/-}$ mice, #*P* = .0130, ###*P* = .0008; WT vs *TWEAK*$^{-/-}$ mice, *****P* < .0001, ****P* = .0006, ***P* = .0020, **P* = .024 (WT mice, n = 10; *Fn14*$^{-/-}$ mice, n = 10; *TWEAK*$^{-/-}$ mice, n = 9). **C,** Schematic diagram of PSA evaluation. *i.v.*, Intravenous. **D,** Movements plus stereotype recordings. Two-way ANOVA followed by the Bonferroni test: WT vs *Fn14*$^{-/-}$ mice, #*P* < .03; WT vs *TWEAK*$^{-/-}$ mice, *****P* < .0001, ****P* = .0001, ***P* = .0057 (WT mice, n = 7; *Fn14*$^{-/-}$ mice, n = 5; *TWEAK*$^{-/-}$ mice, n = 4). **E,** Soluble IgE and mMCP-1 production. One-way ANOVA was performed, followed by a Bonferroni test. Soluble IgE: WT Control vs WT PSA, ****P* = .0003; *TWEAK*$^{-/-}$ Control vs *TWEAK*$^{-/-}$ PSA and *Fn14*$^{-/-}$ Control vs *Fn14*$^{-/-}$ PSA, *****P* < .0001 (control mice: WT mice, n = 7; *Fn14*$^{-/-}$ mice, n = 8; *TWEAK*$^{-/-}$ mice, n = 6; mice with PSA: WT mice, n = 7; *Fn14*$^{-/-}$ mice, n = 6; *TWEAK*$^{-/-}$ mice, n = 6). mMCP-1: WT Control vs WT PSA, ***P* = .0057; *TWEAK*$^{-/-}$ Control vs *TWEAK*$^{-/-}$ PSA, *****P* < .0001; and *Fn14*$^{-/-}$ Control vs *Fn14*$^{-/-}$ PSA, ****P* = .005 (control mice: WT mice, n = 4; *Fn14*$^{-/-}$ mice, n = 3; *TWEAK*$^{-/-}$ mice, n = 5; PSA: WT mice, n = 7; *Fn14*$^{-/-}$ mice, n = 8; *TWEAK*$^{-/-}$ mice, n = 7). **F,** TNF-α, IL-6, PAF, and NO production in mice with PSA. One-way ANOVA followed by Bonferroni tests were performed for WT, *Fn14*$^{-/-}$, and *TWEAK*$^{-/-}$ mice with PSA. TNF-α, IL-6, and PAF show nonsignificant differences. NO: WT vs *Fn14*$^{-/-}$, **P* = .0361; WT vs *TWEAK*$^{-/-}$, **P* = .0287 (n = 4-8 per each group). *mMCP-1*, Mouse mast cell protease-1.

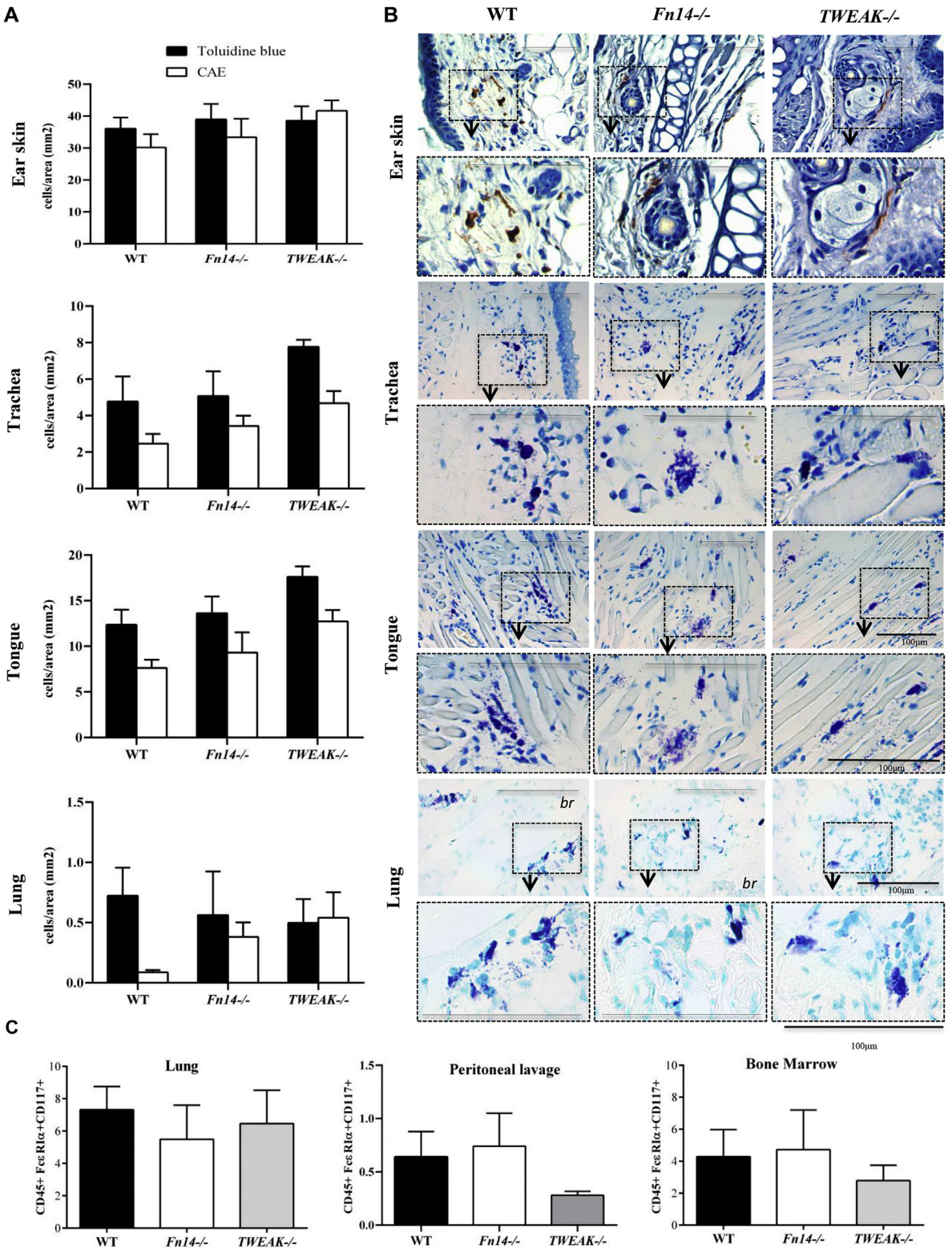


FIG 3. MC characterization of WT, *TWEAK*$^{-/-}$, and *Fn14*$^{-/-}$ mice with PSA. **A,** Quantification of positive cells for toluidine and chloroacetate esterase *(CAE)* staining per area of tissue. Ear skin, trachea, and tongue: WT PSA, 6; *Fn14*$^{-/-}$ PSA, 6; and *TWEAK*$^{-/-}$ PSA, 6. Lung: WT PSA, 8; *Fn14*$^{-/-}$ PSA, 7; and *TWEAK*$^{-/-}$ PSA, 8. One-way ANOVA followed by Bonferroni tests were not significant for both type of staining. **B,** Representative images of CAE (ear skin) or toluidine blue (trachea, tongue, and lung) staining cross-sections of mice with PSA mice at magnifications of 40 × (ear skin, trachea, and tongue) and 63 × (lung) and 100% zoom of the labeled areas. *Scale bars* = 100 μm. **C,** Percentage of CD45^{+}FcεRIα^{+}CD117^{+} cells measured in lung, peritoneal lavage, and bone marrow samples from mice with PSA (n = 5-6 mice per genotype). One-way ANOVA followed by Bonferroni test results were nonsignificant.

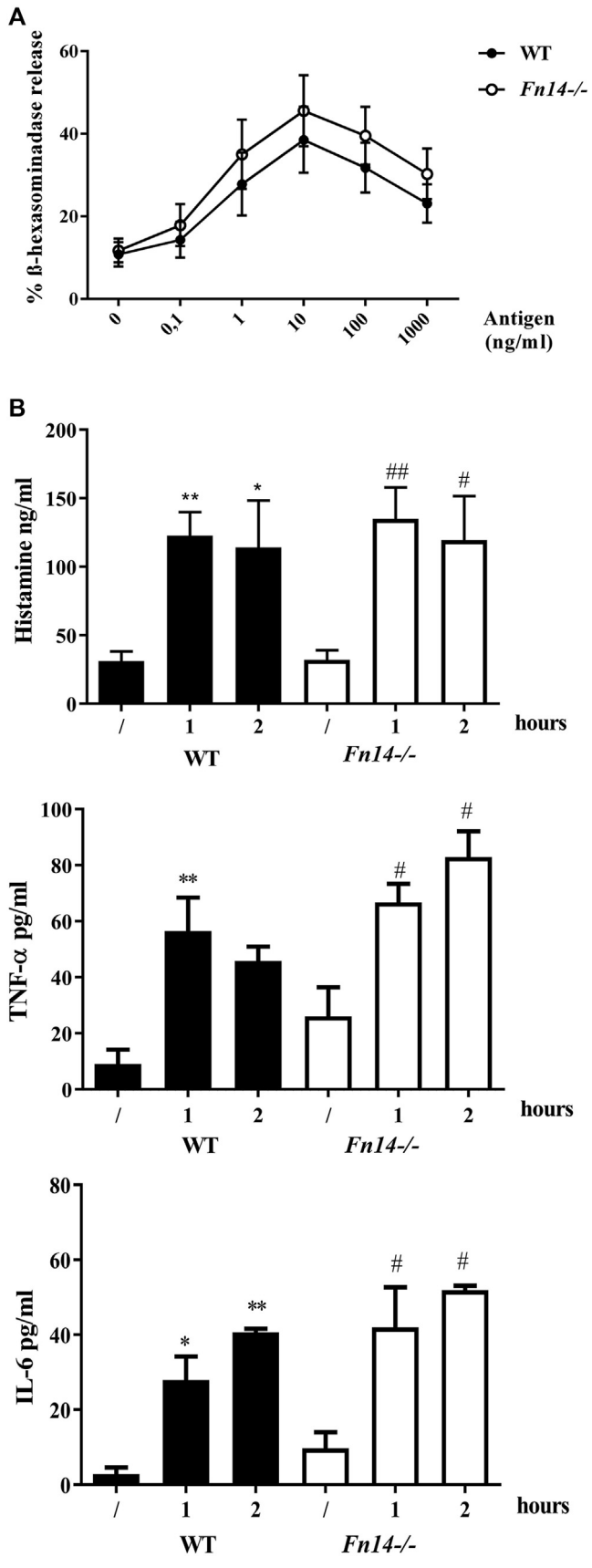


FIG 4. Fn14 does not play a role in BMMC degranulation or mediator release. **A,** BMMCs were sensitized with 100 ng/mL anti-DNP IgE after a range of DNP concentrations. Graphic representation of percentage β-hexosaminidase release (n = 6). **B,** BMMCs were sensitized with anti-DNP IgE (100 ng/mL) and stimulated with DNP-BSA (10 ng/mL) for 1 to 2 hours. Histamine, TNF-α, and IL-6 levels were measured in BMMC supernatants. One-way ANOVA followed by the Bonferroni test: $^{**}P = .0027$ and $^{*}P = .0168$ vs unstimulated WT BMMCs; $^{\#\#}P = .0029$ and $^{\#}P = .0260$ vs unstimulated $Fn14^{-/-}$ BMMCs (n = 7); TNF-α: $^{**}P = .0083$ vs unstimulated WT BMMCs, $^{\#}P < .02$ vs unstimulated $Fn14^{-/-}$ BMMCs (n = 5); IL-6: $^{*}P = .0106$ and $^{**}P = .0041$ vs unstimulated WT BMMCs, $^{\#}P < .04$.

induced vascular permeability in WT MAECs. However, endothelial *Fn14* deficiency prevented the barrier breakdown induced by activated BMMCs (WT or $Fn14^{-/-}$) in MAECs, suggesting an Fn14-dependent effect in the vasculature. Additionally, $TWEAK^{-/-}$ BMMCs were cocultured with WT endothelial monolayers, resulting in a significant reduction in leakage compared with WT BMMCs (Fig 5, *G*). Altogether, these data indicate that TWEAK released by MCs regulates vascular permeability and that Fn14 expression in ECs is essential to induce endothelial barrier breakdown.

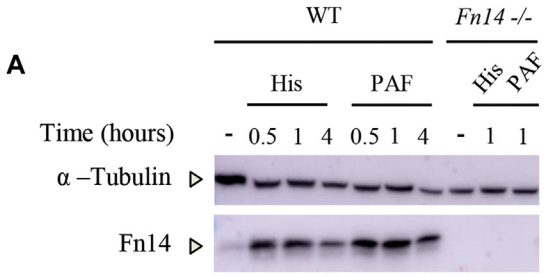
The TWEAK/Fn14 axis participates in *in vivo* systemic and local vascular permeability induced by histamine and PAF

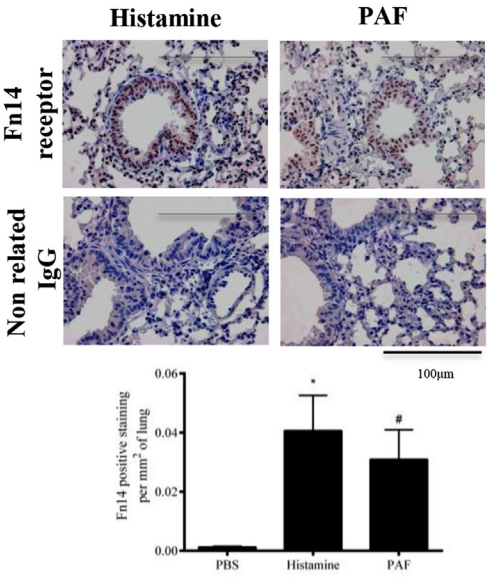
To further analyze the *in vivo* effects of TWEAK and the Fn14 receptor in leakage, we used WT, $TWEAK^{-/-}$, and $Fn14^{-/-}$ mice to evaluate the effect exerted by PSA and subcutaneous injection of histamine and PAF using the Miles assay. Evans blue was administrated with the challenge, and mice with PSA were observed and photographed for 30 minutes. Both $TWEAK^{-/-}$ and $Fn14^{-/-}$ Evans blue–perfused mice with PSA showed less extravasation than the WT mice in their mouths and paws (Fig 6, *A* and *B*). In addition, leakage, as measured based on Evans blue absorbance in dorsal skin punches, was markedly increased in WT mice 15 minutes after injection of histamine or PAF at both low (5 ng/mL) and high (50 ng/mL) doses. However, the extravasation observed in response to these mediators was significantly less in the skin of $TWEAK^{-/-}$ and $Fn14^{-/-}$ mice (Fig 6, *C-E*), supporting the role played by the TWEAK-Fn14 axis in vascular permeability *in vivo*.

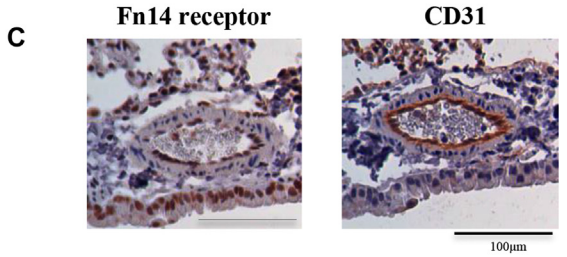
WT mice were treated with anti-TWEAK mAb or control IgG 1 day before the Miles assay was performed to analyze the protective effect of TWEAK-based therapy against vascular permeability induced by histamine or PAF (Fig 6, *F*). Photographs showing the leakage induced by histamine or PAF at different doses revealed clearly decreased extravasation in those mice previously treated with anti-TWEAK compared with IgG-treated mice (Fig 6, *G*). Evans blue determinations demonstrated that histamine/PAF-induced permeability is reduced in skin punches from anti-TWEAK–treated mice compared with IgG-treated mice (Fig 6, H). In general, these data provide evidence that anti-TWEAK mAb treatment ameliorates the subcutaneous vascular permeability process induced by histamine or PAF *in vivo*.

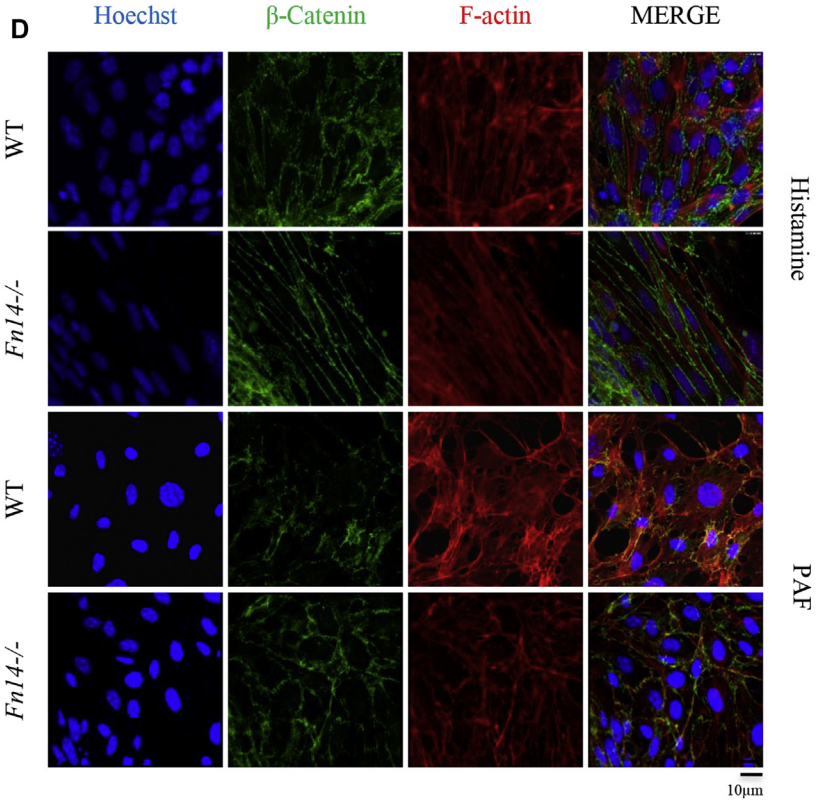
sTWEAK levels are increased in samples from patients with anaphylaxis

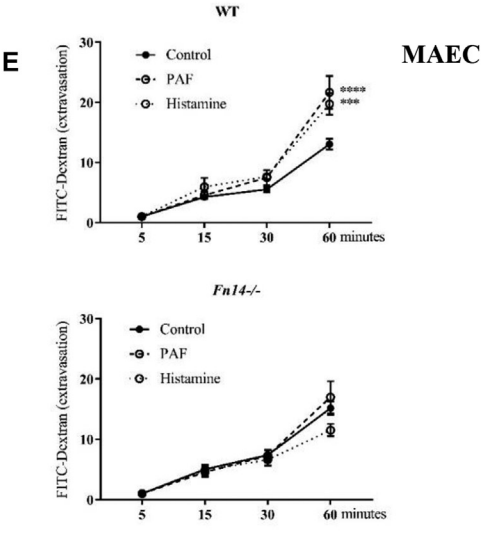
In addition to its mechanistic importance, the TWEAK/Fn14 axis can also be exploited as a long-needed biomarker for human anaphylaxis. Thus we analyzed plasma samples from patients having an anaphylactic reaction to measure sTWEAK levels in

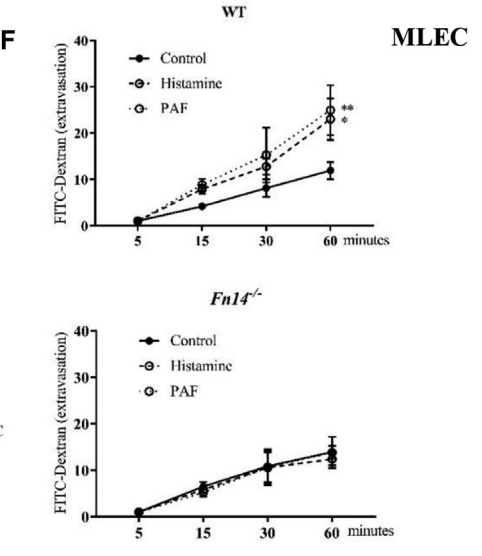

A
WT
Fn14 -/-
His
PAF
His
PAF
Time (hours)
- 0.5 1 4 0.5 1 4 - 1 1
α –Tubulin
Fn14

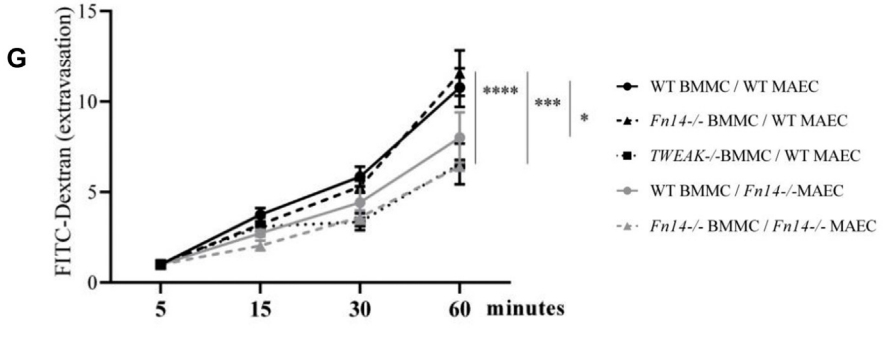

B
Histamine
PAF
Fn14 receptor
Non related IgG
100μm
Fn14 positive staining per mm² of lung
0.06
0.04
0.02
0.00
PBS
Histamine
PAF


C
Fn14 receptor
CD31
100μm


D
Hoechst
β-Catenin
F-actin
MERGE
WT
Fn14-/-
WT
Fn14-/-
Histamine
PAF
10μm


E
MAEC
WT
Control
PAF
Histamine
FITC-Dextran (extravasation)
5
15
30
60 minutes
Fn14-/-
Control
PAF
Histamine
FITC-Dextran (extravasation)
5
15
30
60 minutes


F
MLEC
WT
Control
Histamine
PAF
FITC-Dextran (extravasation)
5
15
30
60 minutes
Fn14-/-
Control
Histamine
PAF
FITC-Dextran (extravasation)
5
15
30
60 minutes


G
FITC-Dextran (extravasation)
5
15
30
60 minutes
WT BMMC / WT MAEC
Fn14-/- BMMC / WT MAEC
TWEAK-/-BMMC / WT MAEC
WT BMMC / Fn14-/-MAEC
Fn14-/- BMMC / Fn14-/- MAEC

comparison with samples from healthy donors. In agreement with our results from mice, an increase in the level of sTWEAK compared with control samples was observed in plasma of samples from those with anaphylaxis (Fig 7, *A*). Individual reactions were graded according to the clinical features described by Brown[39] to examine the correlation between levels of sTWEAK and symptoms of anaphylaxis. The statistical study did not reveal significant differences, indicating no correlation between plasma sTWEAK levels and reaction severity (Fig 7, *B*). Additionally, exposing human dermal microvascular endothelial cells (HMVEC-D) to plasma from patients with anaphylaxis resulted in increased Fn14 expression (Fig 7, *C*).

DISCUSSION

The present study provides both *in vitro* and *in vivo* evidence supporting a role for the TWEAK/Fn14 axis in mice and human subjects with anaphylaxis. *In vivo* animal studies revealed a negative role played by TWEAK/Fn14 interaction in symptoms associated with anaphylaxis and vascular permeability induced by histamine or PAF. This is the first study to report participation by TWEAK/Fn14 in the functional MC/EC microenvironment and provide evidence on the contribution of the endothelial Fn14 receptor to barrier dysfunction.

Anaphylaxis is a life-threatening systemic allergic reaction causing a broad range of symptoms affecting the skin (cutaneous-mucosal), as well as the upper and lower respiratory, gastrointestinal, and cardiac-neurologic systems. Knowledge of the underlying mechanisms involved will determine advances in the future of precision medicine.[40] TWEAK and Fn14 expression and function can vary in tissue and cell dependence and differ between those with acute and chronic disease. However, TWEAK/Fn14 activation plays a pivotally negative role in the pathogenesis of skin inflammation and cardiovascular disease.[14,17] In patients with anaphylaxis, a functional role for the TWEAK/Fn14 axis has not been ruled out, and the only observation reported in the field of allergy indicates an increase of sTWEAK levels in the sputum of children with asthma when compared with healthy control subjects.[23] For different stages of sensitization, our data demonstrate that both PSA and ASA induce TWEAK/Fn14 upregulation in the lung. Although TWEAK expression has been reported to vary in healthy tissues, it seems highly upregulated under pathologic conditions.[41] Our results further reveal that experimental systemic anaphylaxis induces sTWEAK release into the bloodstream and that *TWEAK*$^{-/-}$ and *Fn14*$^{-/-}$ mice had less aggressive symptomatology, a decrease in body temperature, and a decrease in movement and NO release, suggesting that the axis is activated in anaphylactic reactions. In addition, plasma samples from patients with anaphylaxis present greater levels of sTWEAK than those from control subjects and induce Fn14 upregulation in HMVEC-D after *in vitro* incubation.

Because MCs are the main effector cell type involved in anaphylaxis, changes in their functional features could affect anaphylaxis outcomes. Specific toluidine blue and chloroacetate esterase analysis in the ear, trachea, tongue, and lung showed similar numbers between genotypes. In agreement with this, quantification of $CD45^{+}Fc\varepsilon RI\alpha^{+}CD117^{+}$ cell populations supports evidence indicating that the quantity and tissue distribution of MCs are not the cause of the preventive effects observed *in vivo*. *In vitro* analysis was performed in derived BMMCs to investigate plausible functional differences in MCs between genotypes. No significant differences between genotypes were found in either degranulation or key mediators released, such as histamine, IL-6, and TNF-α. In general, these data correlate poorly with the negative role observed for TWEAK/Fn14 in the *in vivo* PSA mouse models, thereby suggesting that the TWEAK/Fn14 axis in MCs is not the main driver of effects observed on the systemic level.

At the cellular level, different growth factors, interleukins, and cytokines positively regulate Fn14 in ECs that participate in inflammatory-immune reactions.[42] Similarly, Fn14 is upregulated in other cellular types, such as vascular smooth muscle cells, cardiomyocytes, macrophages, neurons, and astrocytes, under proinflammatory conditions.[12,43] As occurs in other cellular microenvironments,[43-45] the functional interplay between the receptor and the soluble molecule in different cellular types is of great relevance. In patients with anaphylaxis, relevant mediators, such as tryptase, histamine, or PAF, interact with the vascular endothelium and muscle layers, disrupting the endothelial barrier and modifying essential vascular functions and remodeling the

FIG 5. Loss of Fn14 receptor in MAECs prevents *in vitro* vascular permeability. Endothelial monolayers were stimulated with histamine (1 μmol/L), PAF (0.1 μmol/L), and activated BMMCs. Monolayer integrity was evaluated by using immunofluorescence and TW assays. **A,** Representative immunoblot of Fn14 expression in histamine/PAF-stimulated MAECs (n = 4). **B,** Representative immunohistochemistry images of Fn14 staining in lungs of histamine/PAF-stimulated mice and quantification of Fn14 protein staining per square millimeter of lung: PBS, 5; histamine, 5; and PAF, 4. Student *t* tests were performed versus the PBS group: **P* = .0106 and #*P* = .0118. Representative images of anti-Fn14 receptor and nonrelated IgG staining of lung cross-sections from intravenous histamine- and PAF-treated mice during an hour. *Scale bars* = 100 μm. **C,** Representative images of anti-Fn14 receptor and CD31 staining of lung cross-sections from mice with PSA. *Scale bars* = 100 μm. **D,** Immunofluorescence staining shows β-catenin and F-actin expression in histamine/PAF-stimulated WT and *Fn14*$^{-/-}$ MAECs. Images are representative of 4 separate experiments. **E** and **F,** Quantification of FITC-Dextran molecules extravasated to the TW container and expressed over time. Data represent means of duplicates determined by using TWs in 4 to 8 independent experiments performed on WT and *Fn14*$^{-/-}$ ECs. Two-way ANOVA followed by Bonferroni multiple comparisons tests for each genotype studied: MAEC, *****P* < .0001 vs PAF and ****P* = .0003 vs histamine; MLEC, ***P* = .0051 vs PAF and **P* = .0180 vs histamine. **G,** WT, *Fn14*$^{-/-}$, or *TWEAK*$^{-/-}$ activated BMMCs were incubated on WT or *Fn14*$^{-/-}$ endothelial monolayers. Quantification of FITC-Dextran molecules extravasated to the TW container and expressed over time (n = 4-8). Two-way ANOVA followed by the Bonferroni test: WT BMMC/WT MAEC vs WT BMMC/Fn14$^{-/-}$ MAEC, **P* = .0354; WT BMMC/WT MAEC vs Fn14$^{-/-}$ BMMC/Fn14$^{-/-}$ MAEC, **** *P* ≤ .0001; WT BMMC/WT MAEC vs TWEAK$^{-/-}$ BMMC/WT MAEC, ****P* = .0002; WT BMMC/Fn14$^{-/-}$ MAEC vs Fn14$^{-/-}$ BMMC/WT MAEC, **P* = .0120; Fn14$^{-/-}$ BMMC/WT MAEC vs Fn14$^{-/-}$ BMMC/Fn14$^{-/-}$ MAEC, *****P* < .0001; Fn14$^{-/-}$ BMMC/WT MAEC vs TWEAK$^{-/-}$ BMMC/WT MAEC, *****P* < .0001.

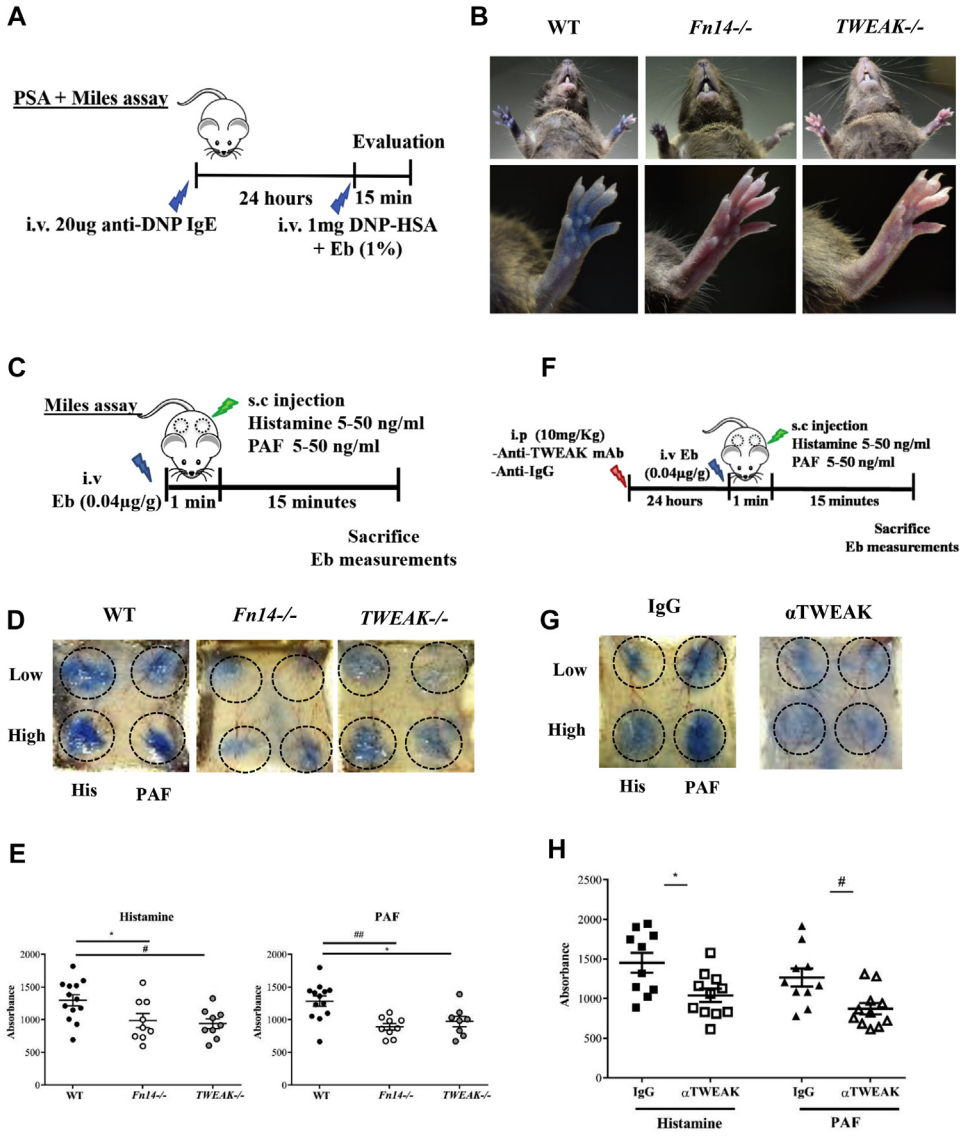
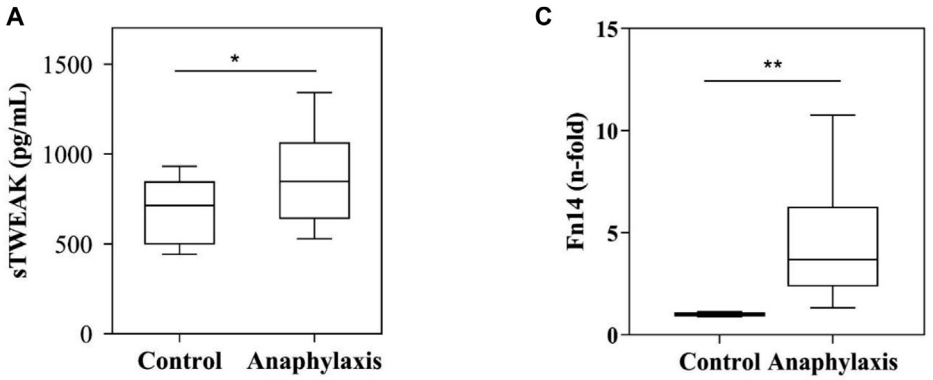


FIG 6. TWEAK/Fn14 genetic deletion or administration of TWEAK blocking antibody prevents histamine/PAF-induced vascular subcutaneous permeability and extravasation associated with PSA. **A,** Schematic diagram of a modified Miles assay combined with the PSA mice model. Mice were challenged together with Evans blue injection. *i.v.*, Intravenous. **B,** Representative pictures of mouths and paws of WT, *Fn14*$^{-/-}$, *and TWEAK*$^{-/-}$ Evans blue–perfused mice with PSA from 3 performed in each genotype. **C,** Schematic diagram of the Miles assay. **D,** Representative Evans blue–stained skin images of WT, *TWEAK*$^{-/-}$, and *Fn14*$^{-/-}$ mice injected subcutaneously with mediators at indicated concentrations. **E,** Quantification of Evans blue release in skin dorsal punches (n = 13 WT, n = 9 *TWEAK*$^{-/-}$, and n = 9 *Fn14*$^{-/-}$ mice per genotype after subcutaneous injection of 20 µL of 50 ng/mL histamine or PAF). One-way ANOVA followed by the Bonferroni test: WT vs *Fn14*$^{-/-}$, **P* = .0409; WT vs *TWEAK*$^{-/-}$, #*P* = .0170 and PAF; WT vs *Fn14*$^{-/-}$, ##*P* = .0013, WT vs *TWEAK*$^{-/-}$, **P* = .0126. **F,** Schematic diagram of modified Miles assay. WT mice were intraperitoneally injected with anti-TWEAK IgG or IgG (10 mg/kg) 24 hours before. *i.p.*, Intraperitoneal; *i.v.*, intravenous. **G,** Representative Evans blue–stained skin images of intraperitoneally injected anti-TWEAK or IgG WT mice and subcutaneously injected with mediators at indicated concentrations. **H,** Evans blue release in response to high doses of histamine and PAF in anti-TWEAK and IgG skin dorsal punches. Mann-Whitney *t* tests versus IgG WT mice (n = 10 mice per group): **P* = .0197, #*P* = .0079.

airways.[5,6] Our *in vivo* and *in vitro* experiments show the effect of histamine and PAF, upregulating Fn14 expression in lung tissue and ECs, and prevention of the barrier breakdown induced by them was observed by specific staining in *Fn14*$^{-/-}$ MAECs. Previous studies demonstrated the role of TWEAK/Fn14 signaling in the integrity of the BBB and its relevance in the central nervous system.[46] Clinical features of anaphylaxis can be associated with structural changes in the bronchial tissue and mechanisms underlying vascular permeability.[33] In the pathogenesis of airway remodeling, changes in EC morphology are fundamental. Variations in E-cadherin and N-cadherin proteins on TWEAK treatment have been described in bronchial ECs.[47] Similarly, other



B

Severity	Mean	SD	Median	P25%	P75%
1	963.9	197.1	949.8	836.3	1076.1
2	871.3	315.8	820.0	636.3	1096.4
3	793.8	288.7	728.4	600.3	946.6

FIG 7. Plasma samples from patients with anaphylaxis present high levels of sTWEAK and induce increased endothelial Fn14 protein expression. **A,** sTWEAK was measured in plasma samples from healthy control donors and patients with anaphylaxis by means of ELISA (Mann-Whitney test vs control samples; n = 24 control samples and n = 55 anaphylactic samples). *P = .01. **B,** Relationship between resulting severity grades and sTWEAK levels was assessed by using the Kruskal-Wallis test (P = .128). **C,** Sera from anaphylactic patients increases endothelial Fn14 receptor expression after 2 hours of incubation in human dermal microvascular endothelial cells (HMVEC-D). Mann-Whitney test vs control samples (n = 4 HMVEC-D, control, n = 9; HMVEC-D, anaphylaxis): **P = .0028.

important adherent and tight junctions have been described as being regulated under TWEAK/Fn14 control in ECs.[37,48,49] The present study characterizes adhesive mechanisms triggered by histamine and PAF through Fn14 in the endothelium during anaphylaxis. Functional studies carried out on transwells (single cultures and cocultures) demonstrated that endothelial Fn14 is key to preventing leakage in the anaphylactic microenvironment. Additionally, coculture experiments indirectly demonstrate the role of sTWEAK as a destabilizing agent of the endothelial barrier when it is released by BMMCs.

The precise mechanism by which the TWEAK/Fn14 axis mediates vascular permeability and anaphylaxis is unclear. Some evidence points to nuclear factor κB activation as one of the main downstream pathways after TWEAK/Fn14 binding. This transcription factor triggers an increase in expression of different cytokines and participates in vascular permeability of the BBB.[45]

Finally, few serologic markers are available when diagnosing anaphylaxis, and the concentration of the main biochemical mediators released by MCs do not always correlate with symptom severity,[4] as has been observed with sTWEAK. Our data point to sTWEAK as a mediator released by MCs in patients with anaphylaxis, although many other inflammatory cells also contribute.[50] Furthermore, we cannot rule out production of sTWEAK in response to histamine and PAF in ECs as well as a possible contribution to a molecular loop. In this sense future studies beyond the scope of ours are necessary. Our *in vivo* data support the notion that either deficiency in *Fn14* or *TWEAK* or systemic TWEAK inhibition by an humanized-TWEAK antibody prevents hyperpermeability in a Miles assay. Although a comprehensive study designed to elucidate the usefulness of sTWEAK in human sera samples from patients with anaphylaxis would be highly beneficial, investigations of this nature face several complications because of the numerous triggers, mechanisms, severities, and the difficulty of obtaining homogeneous samples. Future studies are warranted to identify mediators present in the sera of patients with anaphylaxis that induce Fn14 expression. Nevertheless, we point to sTWEAK and its endothelial Fn14 receptor as new potential strategies to consider in human anaphylaxis.

In conclusion, our findings provide important insights into the role of the TWEAK/Fn14 axis in mouse and human vascular permeability and anaphylaxis.

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Key messages

- ***TWEAK/Fn14*** **genetic deletion decreases PSA and histamine/PAF-induced vascular permeability.**
- **The TWEAK/Fn14 axis participates in endothelial barrier dysfunction mediated during MC/EC interplay.**
- **Plasma samples from patients with anaphylaxis present high levels of sTWEAK and exhibit an increase in endothelial Fn14 protein expression.**

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METHODS

Animal experimental designs

Animal procedures were carried out in accordance with the European Union Directive 2010/63/EU for the care and experimental use of animals. Protocols with reference PROEX: 391/15 received prior approval from the IIS-FJD Ethics Committee.

TWEAK$^{-/-}$ and *Fn14*$^{-/-}$ mice and their WT counterparts were generously provided by Biogen; these have been reported previously and backcrossed onto the C57BL/6 strain.[E1] A group including sham animals (placebo/control) was used in different experimental models, and sensitized but not challenged groups were also used.

To test for PSA, 2-month-old *TWEAK*$^{-/-}$, *Fn14*$^{-/-}$, and WT mice were intravenously injected with 20 μg of anti-DNP IgE (100 μL). After 24 hours, the mice were challenged with an intravenous injection of 1 mg (100 μL) of DNP–human serum albumin to trigger anaphylaxis. A computerized actimeter (Panlab/Harvard) was used to study spontaneous motor activity in PSA mice. The actimeter consists of a transparent cage in which animal activity is measured by using infrared light beams placed in 2 squared frames connected to a computerized control unit. Surface wandering and stereotyped movements were recorded before and each 5 minutes after challenge. Rectal temperatures (taken with a traceable digital thermometer [model VWR I620-2000]) were recorded throughout the time of assessment.

To test for ASA, 2-month-old WT mice were sensitized with an intraperitoneal injection of 1 mg of BSA and 300 ng of pertussis toxin as an adjuvant diluted in saline serum (100 μL). After 14 days, the mice were challenged with intravenous injection of 2 mg of BSA (100 μL). Mice with PSA and ASA were killed 1 hour after challenge by means of cervical dislocation, and blood samples and biopsy specimens (organ collection) were collected for molecular analysis. Whole blood was extracted postmortem by means of cardiac puncture, and serum was obtained. Plasma was used to test serum NO levels (Arbor Assays).

For *in vivo* experiments of vascular permeability, *TWEAK*$^{-/-}$, *Fn14*$^{-/-}$, and WT mice received a subcutaneous injection (50 μL) of histamine and PAF at 5 to 50 ng/mL 10 minutes after intravenous injection with Evans blue dye (0.04 μg/g in NaCl). Systemic permeability was evaluated in the PSA mouse model, adjusting the injection of intravenous Evans blue together with the challenge. Anti-TWEAK antibody or IgG (10 mg/kg) was administered 1 day before the Miles assay. The mouse anti-TWEAK mAb (mIgG2a) is a neutralizing antibody against both murine and human TWEAK. This antibody was generated by immunizing TWEAK-deficient mice on a C57BL/6 background with recombinant human TWEAK and was screened for binding and blocking activity by using standard ELISA methods, flow cytometry, and cell-based functional assays. Mouse anti-TWEAK was used for the first time in the study by Perper et al.[E2]

Skin pieces of these animals were incubated in 500 μL of formamide at 55°C for 48 hours, and the Evans blue content was determined based on absorption at 595 nm. Anti-DNP IgE, DNP–human serum albumin, pertussis toxin, histamine, PAF, and Evans blue were obtained from Sigma-Aldrich.

Histologic analysis

Lungs were fixed by means of perfusion with 4% paraformaldehyde, embedded in paraffin, and prepared in 4-μm paraffin cross-sections for immunohistochemistry, immunofluorescence, and toluidine blue, naphthol AS-D chloroacetate esterase, hematoxylin and eosin, and Masson trichrome staining.

Deparaffinized sections were rehydrated, and antigen retrieval was performed in 10 mmol/L citrate buffer (pH 6). Samples were blocked for 45 minutes with 6% goat serum plus 2% BSA in PBS. Samples were stained with anti-TWEAK (1:50; sc-5558; Santa Cruz Biotechnology, Dallas, Tex), rabbit anti-Fn14 (1:50; 4403S; Cell Signaling, Danvers, Mass), and anti-CD31 (1:50; ab28364; Abcam, Cambridge, United Kingdom). Color was developed with diaminobenzidine (Vector Laboratories, Burlingame, Calif) and then counterstained with hematoxylin, dehydrated, and mounted in DPX (Fluka). Specificity was tested by using a primary antibody with nonrelated IgG. Images were acquired with a Leica DMD 108 microscope (Leica, Wetzlar, Germany), and computerized morphometric analysis was performed with Image-Pro Plus software (version 4.5.0 for Windows; Media Cybernetics, Rockville, Md).

Toluidine blue staining was performed in deparaffinized sections stained with the toluidine blue solution. After washing and drying, samples were mounted with Permount. Naphthol AS-D chloroacetate esterase staining was performed according to the manufacturer's protocol (Sigma-Aldrich).

In vitro murine bone MCs

Murine BMMC generation, isolation, maintenance, and degranulation assays were done, as previously described.[E3]

The degranulation assay was determined based on release of the granule marker β-hexosaminidase in BMMCs cultured on a 96-well plate. Cells were sensitized with anti-DNP IgE (100 or 10 ng/mL) for 24 hours in culture medium to perform degranulation or protein expression BMMC assays. BMMCs (4×10^4 cells) were used for each condition in duplicates. After addition of indicated concentrations of DNP-BSA or vehicle, reaction plates were maintained at 37°C.

In vitro EC cultures

MAECs were isolated from aortas. Two sequential incubations with Dulbecco modified Eagle medium (DMEM)–F12 containing 0.65 mg/mL collagenase type 2 and 4 mg/mL collagenase type 2 for 20 and 40 minutes, respectively, were done, and explants, together with cells, were collected in a plate previously coated with 0.5% sterile gelatin in DMEM-F12 media supplemented with 10 U/mL heparin, 30 μg/mL EC growth factor, 100 U/mL penicillin, 100 g/mL streptomycin, and 15% (vol/vol) heat-inactivated FBS. After several days of outgrowth, MAECs were selected by using a purified rat anti-mouse CD102 antibody after secondary antibody associated with magnetic beads (Dynabeads anti-mouse IgG from the CELLection Pan Mouse IgG Kit; Thermo Fisher). Incubations were performed for 30 minutes at 4°C under constant shaking. All experiments were performed during passages 3 to 7. Before stimulation with histamine (1 μmol/L) and PAF (0.1 μmol/L; Sigma), cells were rendered quiescent by 24 hours of culture in DMEM-F12 and 0.5% FBS.

MLECs were isolated from lungs of mice, as reported previously,[E4] and human dermal microvascular endothelial cells were acquired from Lonza (Basel, Switzerland).

In vitro vascular permeability assays

Endothelial barrier integrity was evaluated by using TWs including a membrane pore size of 0.4 μm (Corning, Corning, NY). WT and *Fn14*$^{-/-}$ ECs were seeded at a density of 10^5 cells/well in TWs previously coated with 0.5% gelatin diluted in sterile water and grown in DMEM-F12 media supplemented as described above. Once confluence had been reached, ECs were FBS starved at 0.5% for 24 hours before experiments. Stimulus or activated BMMCs together with a final concentration of 1 mg/mL FITC-Dextran (Sigma-Aldrich) were added to the upper chamber. Vascular endothelial permeability measurements were determined by measuring the fluorescence of the recipient from the first 5 minutes throughout the study period. All samples were evaluated at least in duplicates.

BMMCs were sensitized with anti-DNP IgE (100 ng/mL) for 24 hours in culture medium to perform *in vitro* BMMC-EC permeability assays. After addition of DNP-BSA (10 ng/mL), 10×10^4 activated cells were added to the upper chambers together with FITC, measuring florescence from 5 to 60 minutes.

Western blot analysis

Cells were lysed with buffer containing Tris, NaCl, EDTA, EGTA, Triton X-100, NP40, protease inhibitors, phenylmethylsulfonyl fluoride, and dithiothreitol. Cellular lysates were shaken for 15 minutes and centrifuged at 12,000 rpm at 4°C. Proteins were separated under reducing conditions on SDS-PAGE gels and transferred to polyvinylidene fluoride membranes.

Protein detection was performed with anti-Fn14 (1:1000; EPR3179; Abcam) and anti-tubulin (1:10000 T5168; Sigma) primary antibodies. After incubation with appropriate HRP-conjugated secondary antibody (Jackson Laboratory, Bar Harbor, Me), proteins were visualized by using ECL Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ). Densitometric analysis of the gels was carried out with ImageJ software (National Institutes of Health, Bethesda, Md).

Immunofluorescence

MAECs from WT and *Fn14*$^{-/-}$ mice were grown in a glass coverslip. Once in confluence, cells were starved in DMEM with 0.5% FBS for 24 hours and stimulated with histamine and PAF for 10 minutes. After stimulation, cells were fixed in 4% paraformaldehyde and stained with anti–β-catenin Alexa Fluor 488 (1/50; Cell Signaling), Texas Red-X phalloidin (Invitrogen, Carlsbad, Calif), and Hoechst for nuclei counterstaining. Immunofluorescence images were acquired by using an inverted confocal microscope (LSM700; Carl Zeiss, Oberkochen, Germany) with a 40 × Plan-Apochromatic oil immersion objective. The confocal microscopic images presented are 2-dimensional maximal projections of a z-series through the cell depth.

Patients

The studied population included 55 blood samples from adults with anaphylaxis (>18 years of age; 65.45% of the population was female) treated at the allergy unit of the Fundación Jiménez Díaz, Hospital Central de la Cruz Roja, Madrid, Spain, and Guadalajara Hospital, Guadalajara, Spain, and 24 control samples from healthy control donors. Triggers suspected to provoke reactions were as follows: drugs (67.27%), foods (21.82%), and others (10.91%). Patients who fulfilled the definition of anaphylaxis according to the 2006 National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network criteria were included.[E5] A serum and plasma sample were taken during the episode, and the criteria of severity was applied according to the grading system based on clinical symptoms by using the Brown classification.[E6] The mild group was grade 1 (21.82% of the population), the moderate group was grade 2 (50.91%), and grade 3 (27.27%) included patients with severe anaphylaxis with cardiovascular involvement and hypotension. Patients were followed up at the clinic, where an allergy work-up was performed as needed (skin prick tests, specific IgE measurements, and/or challenge tests), and the diagnosis of anaphylaxis was confirmed by an allergist.

The protocol was approved by the ethics committee (PIC38/2016_FJD). The authors adhere to the declaration of Helsinki, and patients were included after providing informed consent.

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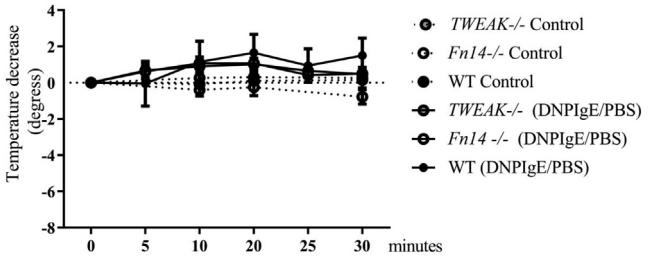

FIG E1. Temperature measurements in sensitized but not challenged and control mice. Measurements were addressed in WT, *TWEAK*$^{-/-}$, and *Fn14*$^{-/-}$ mice over 30 minutes. Results of 2-way ANOVA followed by the Bonferroni test were nonsignificant.

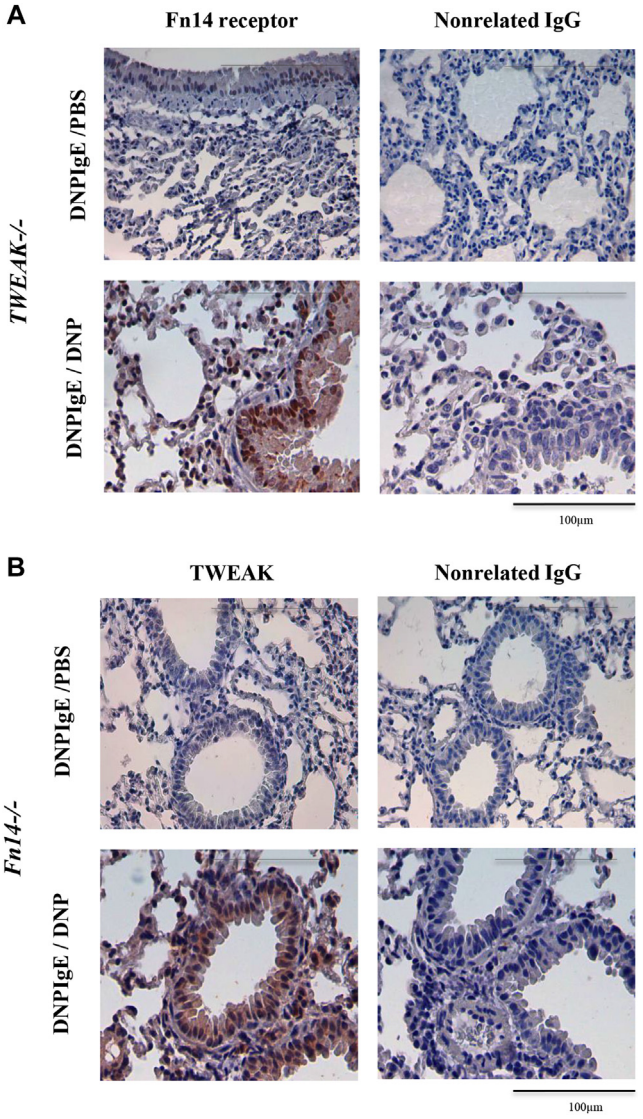

FIG E2. TWEAK or Fn14 receptor levels increase in lung tissues from *Fn14*$^{-/-}$ and *TWEAK*$^{-/-}$ mice, respectively, in the PSA model. Representative images of anti-TWEAK, anti-Fn14, and nonrelated IgG staining of lung cross-sections of mice with PSA. *Scale bars* = 100 μm.

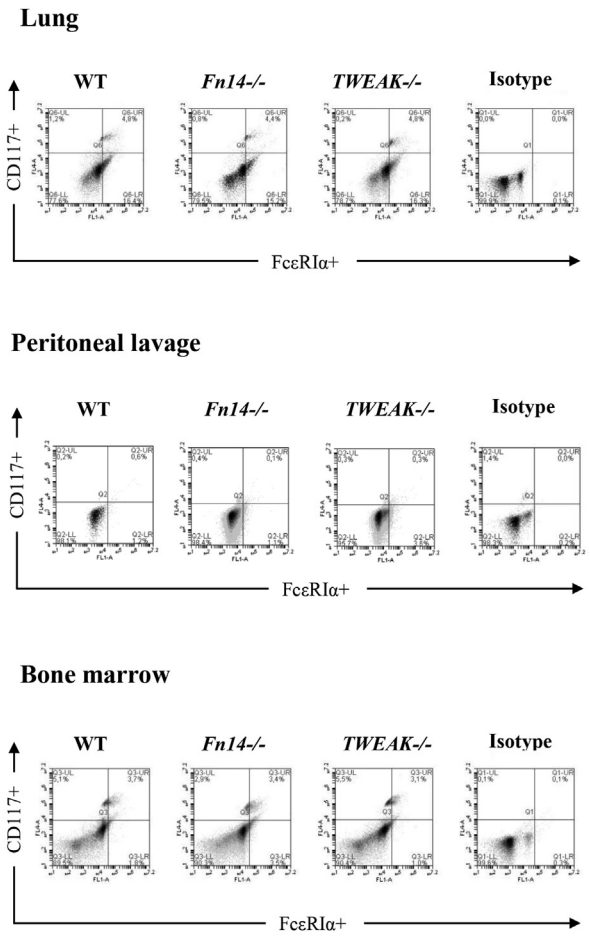

FIG E3. Mast cell characterization of WT, *TWEAK*$^{-/-}$, and *Fn14*$^{-/-}$ mice with PSA. Representative gating strategy shows FcεRIα and CD117 double-positive cell populations of lung **(A)**, peritoneal lavage **(B)**, and bone marrow **(C)** specimens from WT, *TWEAK*$^{-/-}$, and *Fn14*$^{-/-}$ mice. Samples were incubated with MAR-1 FITC mAb and the allophycocyanin/Cy7 anti-mouse CD117, and fluorescence intensity was measured by using flow cytometry. Panels show a representative mouse of each genotype and the corresponding FITC-isotype control.

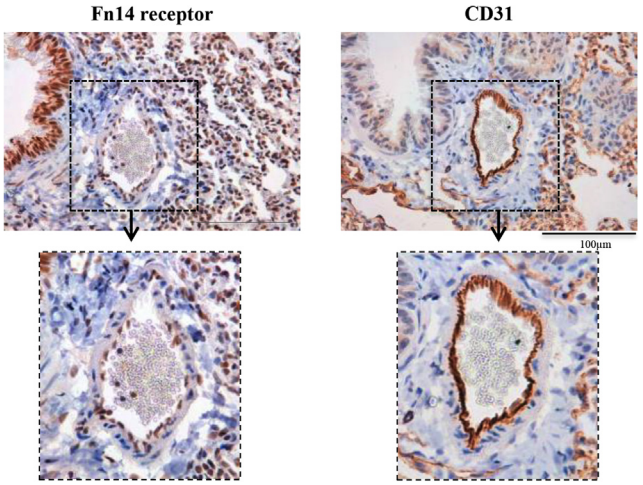

FIG E4. Representative images of anti-Fn14 receptor and CD31 of lung cross-sections from mice with ASA with magnifications of 40 × and 100% zoom of the labeled area. *Scale bars* = 100 μm.