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This information is current as of March 29, 2016.

J Immunol published online 21 March 2016
<http://www.jimmunol.org/content/early/2016/03/18/jimmunol.1501510>

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Topical Application of Fingolimod Perturbs Cutaneous Inflammation

Wai Y. Sun,^{*,†} David P. Dimasi,^{*} Melissa R. Pitman,^{*} YiZhong Zhuang,[†] Robert Heddle,^{†,‡,§} Stuart M. Pitson,^{*,†} Michele A. Grimaldeston,^{*,†} and Claudine S. Bonder^{*,†}

The prevalence of allergies, including rhinitis, eczema, and anaphylaxis, is rising dramatically worldwide. This increase is especially problematic in children who bear the greatest burden of this rising trend. Increasing evidence identifies neutrophils as primary perpetrators of the more severe and difficult to manage forms of inflammation. A newly recognized mechanism by which neutrophils are recruited during the early phase of histamine-induced inflammation involves the sphingosine kinase (SK)/sphingosine-1-phosphate axis. This study examines whether topical application of fingolimod, an established SK/sphingosine-1-phosphate antagonist already in clinical use to treat multiple sclerosis, may be repurposed to treat cutaneous inflammation. Using two mouse models of ear skin inflammation (histamine- and IgE-mediated passive cutaneous anaphylaxis) we topically applied fingolimod prophylactically, as well as after establishment of the inflammatory response, and examined ear swelling, SK activity, vascular permeability, leukocyte recruitment, and production of proinflammatory mediators. The present study reveals that when applied topically, fingolimod attenuates both immediate and late-phase responses to histamine with reduced extravasation of fluid, SK-1 activity, proinflammatory cytokine and chemokine production, and neutrophil influx and prevents ear swelling. Intravital microscopy demonstrates that histamine-induced neutrophil rolling and adhesion to the postcapillary venules in the mouse ears is significantly attenuated even after 24 h. More importantly, these effects are achievable even once inflammation is established. Translation into humans was also accomplished with epicutaneous application of fingolimod resolving histamine-induced and allergen-induced inflammatory reactions in forearm skin. Overall, this study demonstrates, to our knowledge for the first time, that fingolimod may be repurposed to treat cutaneous inflammation. *The Journal of Immunology*, 2016, 196: 000–000.

Inflammation is a finely orchestrated immune response to irritation, infection, or injury and is either acute or chronic in nature. Allergic inflammation is initially an IgE-mediated “immediate” type I hypersensitivity reaction but in clinical disorders is often followed by late IgE responses and some T cell-mediated type IV responses (reviewed in Ref. 1). Allergen avoidance is the cornerstone of management; however, this is extremely difficult given the ubiquitous nature of most allergens. Immunotherapy successfully alters hypersensitivity but it is invasive, costly, and not without risks (2). Patients with chronic symptoms often require

long-term treatment with antihistamines and corticosteroids, which have limited efficiency, and with steroids, which are known to interfere with metabolism and growth (3). The prevalence of allergic inflammation-associated diseases continues to rise across all age, gender, and racial groups. They continue to cause significant impact on comorbidities, with current annual economic costs exceeding U.S. \$300 billion and predicted to rise (4, 5). Clearly, a better understanding of the mechanisms underpinning allergic inflammation is required for new treatment options to become available.

Allergic inflammation is a multistep and progressive disease wherein the early phase includes activation of mast cells and basophils, which release proinflammatory mediators (e.g., histamine) (1). These inflammatory mediators activate the local vasculature for increased surface expression of adhesion molecules that promote the recruitment of eosinophils and neutrophils via slow rolling, tethering, adhesion, and transmigration (6). Eosinophils have long been recognized in allergic inflammation (1). In contrast, neutrophils have been largely ignored despite evidence that 1) sudden-onset fatal asthma correlates not with eosinophilic content but rather with neutrophil number (7, 8), 2) neutrophil recruitment is important for allergic skin inflammation (9), and 3) neutrophils themselves are a major source of histamine and often accumulate in numbers far greater than other histamine-producing cells (e.g., mast cells) (10, 11). Furthermore, with the lifespan of some neutrophils recognized as 3–5 d (12) as opposed to the previously accepted 6–8 h (13), and demonstration that these cells can also be reprogrammed into subpopulations (14), fundamental misconceptions about neutrophil biology are being recognized and re-evaluated.

Sphingosine kinase (SK) is a highly conserved lipid enzyme in the sphingomyelin pathway that catalyzes the phosphorylation of sphingosine to form sphingosine-1-phosphate (S1P) (15). Two

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Received for publication July 6, 2015. Accepted for publication February 23, 2016.

This work was supported by Australian National Health and Medical Research Council project grants to C.S.B., S.M.P., and M.A.G., a Heart Foundation fellowship to C.S.B., an Australian National Health and Medical Research Council senior research fellowship to S.M.P., an Australian National Health and Medical Research Council career development fellowship to M.A.G., a Royal Adelaide Hospital Research Foundation early career fellowship to M.R.P., and a Cooperative Research Centre biomarker translation scholarship to W.Y.S.

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The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; EPH, ethanol, propylene glycol, and H₂O; HMEM, Hanks' MEM; HSA, human serum albumin; PCA, passive cutaneous anaphylaxis; SK, sphingosine kinase; SKi, sphingosine kinase inhibitor; S1P, sphingosine-1-phosphate; SPT, skin prick test; WT, wild-type.

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isoforms of SK (SK-1 and SK-2) have been identified, cloned, and characterized (16), and they differ in subcellular localization patterns, distribution in adult tissue (15, 16), and have both overlapping and distinct biological functions (17–19). Mice lacking either SK gene (*Sphk1* or *Sphk2*) are viable, fertile, and phenotypically normal (20, 21), but deletion of both genes causes embryonic lethality due to severe defects in angiogenesis and neurogenesis (21). Increasing evidence suggests that maintaining S1P homeostasis is important for preventing unwanted immune responses (22–24) and to retain appropriate vascular barrier integrity (25). Although the basal level of intracellular S1P is generally low, biological stimuli (e.g., histamine and TNF- α) can increase its production via transient activation of ERKs 1 and 2 and SK (17). Extracellular S1P binds to a family of G protein-coupled receptors (S1P_{1–5}) that induce downstream signaling, such as PI3K/Akt and ERK-1/2 (26) with S1P_{1–3} expressed on endothelial cells (27, 28). With SK/S1P increased in inflammatory-based diseases (25, 29–32) and *Sphk1* knockout mice exhibiting attenuated inflammatory responses (29, 33), a fundamental role for the SK/S1P axis in this disease is beginning to emerge, and targeting it directly represents a feasible therapeutic strategy.

Fingolimod (known as FTY720 or Gilenya) is a structural analog of sphingosine that is phosphorylated by SK [predominantly SK-2 (34)] to act initially as an agonist of the S1P receptors (S1P_{1,3–5}) and then as an antagonist by internalizing S1P₁ on lymphocytes to prevent their egress from secondary lymphoid organs (35, 36). Based on these lymphopenia-like properties, fingolimod gained approval as an orally active immunomodulatory drug for the treatment of relapsing and remitting multiple sclerosis by U.S. Food and Drug Administration and Australian Therapeutic Goods Administration. Recent studies have revealed that fingolimod can also inhibit S1P production via degradation of the SK-1 protein (37).

We recently demonstrated that fingolimod attenuated histamine-induced neutrophil rolling along HUVEC under shear stress and that i.p. injection of fingolimod perturbed histamine-induced neutrophil recruitment to the cremaster muscle (29). A role for SK in inflammation is further supported by fingolimod and other sphingosine analogs attenuating airway hyperresponsiveness and inflammation in mouse models of asthma (38, 39). Under these premises, we aimed to determine whether fingolimod could be applied epicutaneously to perturb inflammation in mouse skin when administered either prophylactically or after establishment of the inflammatory reaction. Successful responses led us to determine the contributing factors that underpin this anti-inflammatory effect. Finally, we investigated whether fingolimod could effectively prevent and treat histamine- and allergen-induced inflammation in the skin of humans.

Materials and Methods

Study approvals

All mouse experimental procedures were approved by the Animal Ethics Committees of SA Pathology and the University of Adelaide as per the guidelines established by the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*. Human studies were given ethical clearance from the Royal Adelaide Hospital Human Ethics Committee (Adelaide, SA, Australia) with informed written consent obtained in accordance with the Declaration of Helsinki.

Mice

Wild-type (WT), SK-1 knockout (*Sphk1*^{−/−}), and SK-2 knockout (*Sphk2*^{−/−}) female and male mice on a C57BL/6 background (20, 21) were housed under pathogen-free conditions at SA Pathology and used between 8 and 12 wk of age.

Histamine-induced inflammation of the ear

Mice were anesthetized by i.p. injection of ketamine (10% [v/v], Ceva Group, Glenorie, NSW, Australia) and xylazine (5% [v/v], Ceva Group) in 0.9% saline (10 μ l/g) prior to being injected intradermally with histamine

(Sigma-Aldrich, St. Louis, MO) at 0.2, 2, or 8 mg in Hanks' MEM (HMEM)–PIPES (20 μ l, Sigma-Aldrich) to the right ear and vehicle controls (HMEM–PIPES, 20 μ l) to the left ear. Change in ear thickness from baseline was measured at 15, 30, 60, 120, 180, 240, 300, and 360 min postinjection with a dial thickness gauge (model G-1A, Ozaki Manufacturing, Tokyo, Japan).

For systemic prophylactic treatment, anesthetized mice were retro-orbitally (i.v.) injected with fingolimod at 0.5 mg/kg (Cayman Chemical, Ann Arbor, MI) in 100 μ l saline per mouse or vehicle control (saline, 100 μ l). Twenty-four hours after treatment, 8 mg histamine or vehicle controls in 20 μ l HMEM–PIPES was injected intradermally to the right and left ears, respectively. Ear thickness was measured as detailed above.

For prophylactic treatment, anesthetized mice received a single topical application of SK inhibitors (SKi; SKI-II; Cayman Chemical) (40), MP-A08 (41), or fingolimod, all at 10 μ g in 40 μ l surfactant (100% ethanol, propylene glycol, and H₂O [EPH] at 2:1:1 [v/v/v]) (42) to both ears, and separate control groups received vehicle controls (EPH). Twenty-four hours after application, histamine challenge and measurement of ear thickness were performed as described above.

Alternatively, mouse ears were intradermally injected with 8 mg histamine in 20 μ l HMEM–PIPES followed 30 min later by topical application of fingolimod (10 μ g) or vehicle (EPH) to both ears and ear thickness was measured over time.

IgE-mediated passive cutaneous anaphylaxis

Anesthetized mice were injected intradermally with anti-DNP IgE mAb (clone SPE-7) at 5 μ g/ml in 20 μ l HMEM–PIPES to the right ear and vehicle control (HMEM–PIPES) to the left ear (42). Sixteen hours following IgE sensitization, mice were injected i.v. (retro-orbital) with 2 mg/ml DNP–human serum albumin (HSA) (Sigma-Aldrich) in 100 μ l sterile 0.9% saline. Epicutaneous application of fingolimod at 10 μ g in EPH (40 μ l) was performed at 30 min after DNP–HSA injection. Ear thickness was measured over time.

SK activity assay

For SK-1 activity, whole-ear lysates were digested with Liberase (Roche, Castle Hill, NSW, Australia) at 37°C for 2 h prior to separation of tissue debris with a 40- μ m cell strainer (BD Biosciences, North Ryde, NSW, Australia). Lysates were incubated with D-erythro-sphingosine (Cayman Chemical) solubilized in 0.05% Triton X-100 and γ -[³²P]ATP (PerkinElmer, Melbourne, VIC, Australia). For SK-2 activity, ear lysates were prepared in buffer containing 1 M KCl and incubated with D-erythro-sphingosine solubilized in BSA/PBS and γ -[³²P]ATP. The radioactively labeled S1P was resolved by 2 thin-layer chromatography separations in the solvents containing butanol, ethanol, water, and acetic acid (8:2:2:1). The radioactive spots were quantified by phosphorimaging with Typhoon 9410 (Beckman Coulter, Fullerton, CA) and ImageQuant 5.2 program (GE Healthcare, Rydalmere, NSW, Australia). SK activity is defined as arbitrary radioactive spot intensity units per milligram protein of ear extract.

Measurement of S1P formation

The rate of S1P formation in intact VeraVec cells (hVera101, human VeraVec HUVEC, Angiocrine Bioscience, New York, NY) was determined using an *in situ* assay of SK activity. Briefly, VeraVec cells were seeded at 70% confluence (in 20-cm² culture dishes) and changed to serum-free media for 1 h prior to labeling, at which time inhibitor treatments (vehicle or 100 nM FTY720) were added. Cells were then labeled with 0.5 μ Ci [³H]sphingosine (PerkinElmer, Rowville, VIC, Australia), and histamine at 25 μ M (or vehicle) was added and the cells were incubated for 30 min. The cells were then washed with cold PBS and harvested by scraping. [³H]S1P formed during the 30-min incubation was then extracted from the cells via a modified Bligh–Dyer extraction (43). Briefly, cell pellets were resuspended in 300 μ l acidified methanol (100:1, methanol/concentrated HCl) and sonicated for 30 s. To this 300 μ l 2 M KCl, 300 μ l chloroform, and 30 μ l 3 M NaOH were added. After vigorous mixing, a phase separation enabled separation of S1P in the upper aqueous methanol phase from sphingosine in the lower chloroform phase. The [³H]S1P in the aqueous methanol phase was then analyzed by scintillation counting. All analyses were performed in triplicate and corrected for total cell number.

Flow cytometric analysis

Whole ears were harvested, digested with Liberase at 37°C for 2 h, and then filtered with a 40- μ m cell strainer (BD Biosciences) to remove debris. Following FcR blocking, cells were incubated with anti-Gr1-PE (clone RB6-8C5; granulocyte marker) and anti-F4/80-FITC (clone BM8; pan macrophage marker) Abs or IgG isotype controls (all at 2 μ g/ml from

eBioscience, San Diego, CA) on ice for 30 min prior to analysis using an Accuri flow cytometer (BD Biosciences) and FCS Express 4 flow cytometry (research edition) software (De Novo Software, Los Angeles, CA).

Whole blood was collected from anesthetized mice followed by RBC lysis using ACK lysis buffer. Cells were incubated with CD3-FITC or IgG isotype control Abs (2 μ g/ml, eBioscience) on ice for 30 min prior to analysis using the Accuri flow cytometer and FCS Express 4 flow cytometry (research edition) software.

The inflammatory cytokine assay used a cytometric bead array kit as per the manufacturer's protocol (BD Biosciences). Briefly, whole ears were harvested, digested, and filtered to remove debris (as detailed above) prior to incubation with the microbeads that conjugated to cytokines (IL-6, IL-10, MCP-1, IFN- γ , TNF, IL-12p70, and KC [homolog of human IL-8]). Sample analysis was performed using an Accuri flow cytometer with a selectable laser module (BD Biosciences) and FCAP Array software (version 3.0, BD Biosciences).

Histology

Ear pinnae were fixed in 10% (v/v) buffered formalin at 4°C overnight and then embedded in paraffin. Four-micrometer cross-sections were prepared for H&E staining. Analysis was performed using an Olympus CX41 microscope (Hachioji-shi, Tokyo, Japan), and images were captured using NanoZoomer digital slide scanner (NDP-Hamamatsu, Hamamatsu, Japan).

Intravital microscopy and in vivo experimental procedure

Intravital microscopy to measure extravasation of fluid in the mouse ear vasculature was performed as previously described (44). Mice were anesthetized as above before 10 μ g fingolimod or diluent (EPH) was topically applied to an ear. Twenty-four hours later the mice were injected intraorbitally (i.v.) with 100 μ l 10 mg/ml FITC-dextran (150 kDa) followed immediately by their ears being intradermally injected with either 8 mg histamine or vehicle control (HMEM-PIPES, 20 μ l). The ear was then placed over a raised platform, mounted under a glass coverslip, and positioned under a $\times 20$ objective with the whole mouse being contained within a heated chamber of an LSM 710 two-photon microscope (Carl Zeiss, Jena, Germany) for imaging. The FITC-dextran was excited using a tunable Mai Tai Ti:Sapphire multiphoton laser (Spectra-Physics, Santa Clara, CA) and external nondescanned detectors were used to capture the

fluorescence signal. A stack of three images over a range of 10 μ m was then acquired every 5 min over the course of 15 min using Zen 2011 (version 7.0.4.0, Carl Zeiss). Image analysis was undertaken using a macro written for use within Image J (45). As all images were in color, the green channel was extracted and then a median filter with a radius of 2.0 pixels was employed to reduce noise. A fluorescence threshold was then manually applied by the user to the time 0 image, with subsequent images in the series then using the threshold values from the time 0 image. Image analysis then determined the percentage area covered by the threshold region.

To examine leukocyte trafficking, histamine was injected intradermally into the ear and fingolimod (or diluent) was applied topically 30 min thereafter. A separate group of histamine-treated mice received epicutaneous application of 0.5% antihistamines (chlorpheniramine and cimetidine; 0.2 mg in 40 μ l EPH; Sigma-Aldrich) at 30 min after histamine challenge. Mice were retro-orbitally (i.v.) injected with FITC-dextran (150 kDa, 1 mg in 100 μ l PBS, Sigma-Aldrich) and rhodamine 6G (1 mg in 100 μ l PBS, Sigma-Aldrich). Of note, both chlorpheniramine and cimetidine were confirmed functionally active in histamine-induced intracellular calcium influx experiments (data not shown). Intravital microscopy of these mouse ears was performed with a $\times 20$ objective lens and $\times 10$ eyepiece. Live images were recorded every 2 s for up to 4 min using Zen 2011. Two postcapillary venules per mouse ear were analyzed for leukocyte trafficking by playback analysis. Leukocyte rolling was defined as slow rolling along the blood vasculature whereas adherent leukocytes were defined as those remaining stationary for >10 s.

Human skin prick test and epicutaneous application of fingolimod

Thirteen healthy adults (five females, eight males; age range, 28–63 y) consented to a routine allergy skin prick test (SPT) (46) with fingolimod treatment in the Clinical Immunology Unit of the Royal Adelaide Hospital. Because of the adverse events ascribed to fingolimod (47), contraindications for participants were recent (<6 mo) occurrence of myocardial infarction, unstable angina, recent cardiac arrhythmia, stroke, transient ischemic attack, heart failure, risk of pregnancy, and systemic infection. Furthermore, to identify and exclude any participants with a predisposition for side effects, baseline blood pressure, electrocardiogram, full blood examination, and liver function test were undertaken prior to the study. A

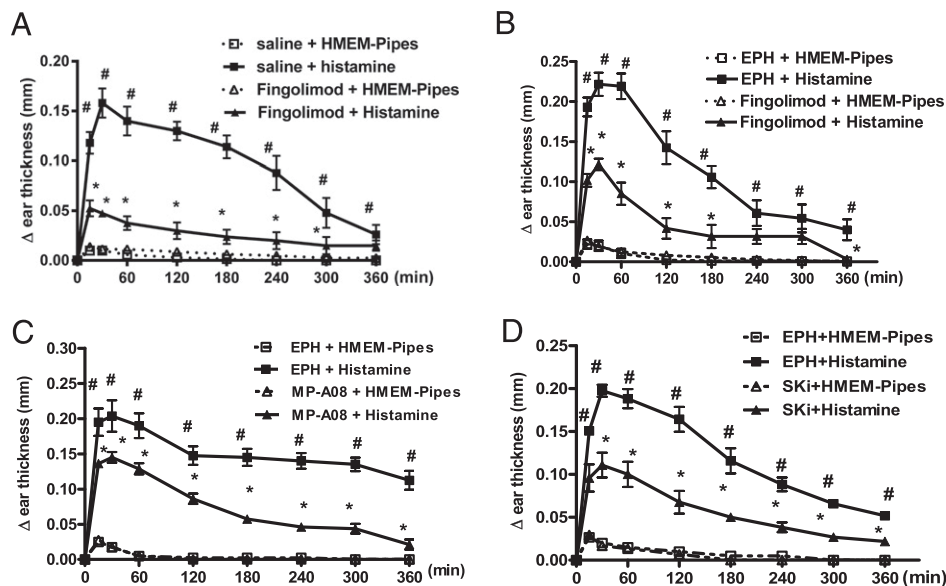


FIGURE 1. Systemic and topical prophylactic treatment of SK inhibitors attenuates histamine-induced ear swelling. **(A)** Fingolimod (0.5 mg/kg) or saline (control) was injected retro-orbitally 24 h prior to histamine (8 mg) and HMEM-PIPES (control) intradermal injection to the right and left ears, respectively. Changes (Δ) in ear thickness were measured; $n = 7$ –8 mice/group, in four individual experiments. $^{\#}p < 0.05$ versus saline + HMEM-PIPES, $^{*}p < 0.05$ versus saline + histamine. **(B)** Fingolimod (10 μ g) or EPH (control) was applied topically to the ears 24 h prior to histamine (8 mg) and HMEM-PIPES intradermal injection and ear thickness measurements; $n = 9$ mice/group, in three individual experiments. $^{\#}p < 0.05$ versus EPH + HMEM-PIPES, $^{*}p < 0.05$ versus EPH + histamine. **(C)** MP-A08 (10 μ g) or EPH was applied topically to the ears with a regimen of three applications during 24 h prior to histamine (8 mg) and HMEM-PIPES intradermal injection and ear thickness measurements; $n = 6$ mice/group, in three individual experiments. $^{\#}p < 0.05$ versus EPH + HMEM-PIPES, $^{*}p < 0.05$ versus EPH + histamine. **(D)** SKi (10 μ g) or EPH was applied topically to the ears 24 h prior to histamine (8 mg) and HMEM-PIPES intradermal injection and ear thickness measurements; $n = 9$ mice/group, in three individual experiments. $^{\#}p < 0.05$ versus EPH + HMEM-PIPES, $^{*}p < 0.05$ versus EPH + histamine.

repeat electrocardiogram at 6 h and repeat blood tests at 7 d were performed.

Using established standard operating procedures (46), a small amount of histamine, cat dander, *Alternaria* (mold), *Dermatophagoides pteronyssinus* (dust mite), and rye grass pollen were administered into the inner forearm skin (approximately midway along the volar aspect) of both participant arms. The wheal and flare responses were measured at 10-min intervals for a minimum of 60 min by an investigator blinded to the treatments applied. Using published methods (48), 10 and 20 min after challenge, fingolimod (0.0125 mg in 20 μ l EPH) was applied topically at the skin prick site of histamine and the most reactive allergen on one forearm whereas the other forearm received 20 μ l of the diluent (EPH) only to the same test sites. This dose of fingolimod is 20-fold less than that shown to cause transient bradycardia in humans via oral administration (49).

Statistical analysis

Data are shown as means $\pm$ SEM from at least three independent experiments. For animal experiments, results were statistically analyzed by two-way ANOVA with the Bonferroni posttest for repeated measures and multiple comparisons. For in vitro experiments, results were analyzed by an unpaired Student *t* test or a one-way ANOVA with a Dunnett posttest. For the human study, a two-way ANOVA with the Bonferroni posttest for repeated measures and multiple comparisons was used and collectively shown are the means $\pm$ SEM for each time point of the 8–13 participants. A *p* value <0.05 was considered significant.

Results

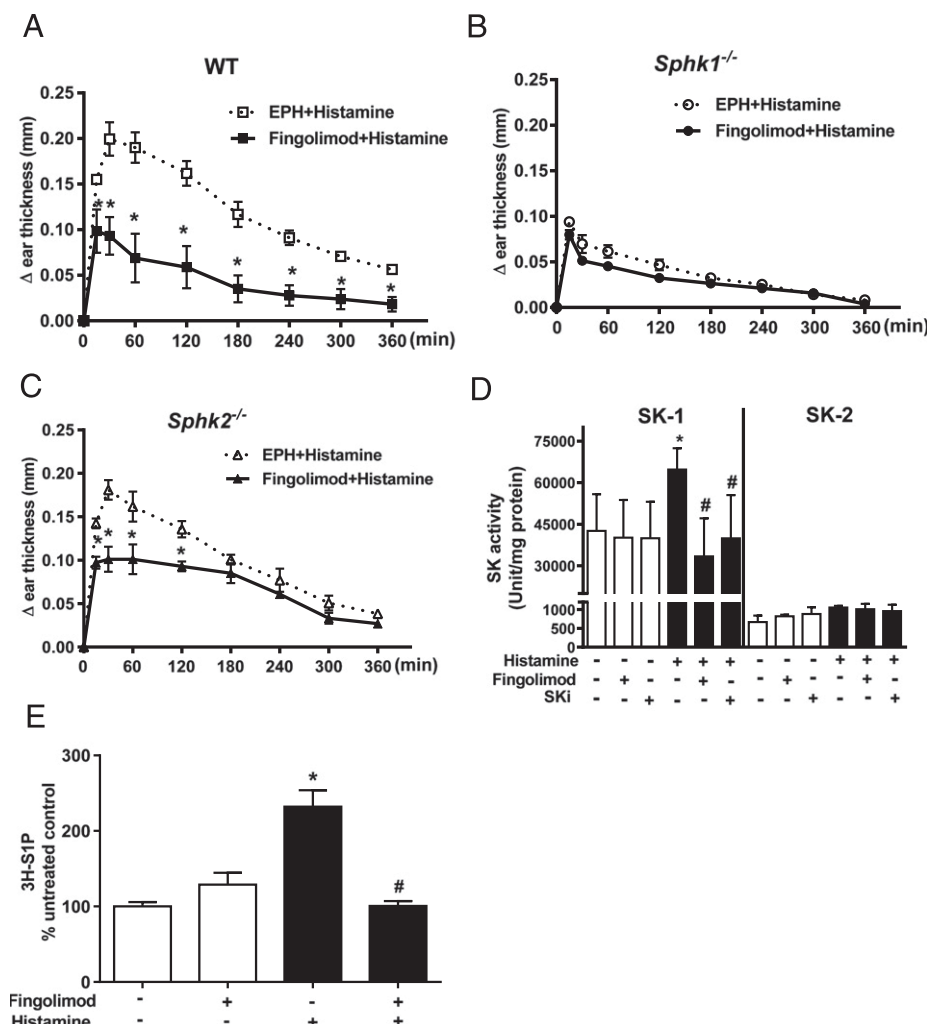
Prophylactic treatment of SK inhibitors attenuates histamine-induced inflammation

As SK and S1P can regulate the expression of adhesion molecules on vascular endothelium and activate inflammatory cells (29, 50,

51), we hypothesized that SK inhibitors (e.g., fingolimod, SKI, and MP-A08) may be used to treat inflammation. First, we optimized histamine-induced inflammation in the ears of C57BL/6 mice and determined that whereas 0.2, 2, and 8 mg histamine significantly increased ear swelling within 30 min, the 8 mg resulted in the most profound swelling, which was sustained over 6 h (see Supplemental Fig. 1) and was thus used for all subsequent experiments. To examine the effect of SK inhibitors on histamine-induced ear swelling we administered 0.5 mg/kg fingolimod or vehicle control (saline) via i.v. injection 24 h prior to 8 mg histamine challenge. As shown in Fig. 1A, histamine-induced ear thickness was significantly attenuated in mice pretreated with fingolimod. We next investigated whether fingolimod can be applied topically to inhibit this inflammatory response. Fig. 1B shows that mice given an epicutaneous application of 10 μ g fingolimod on the ear 24 h prior to 8 mg histamine challenge demonstrated a significant reduction in ear swelling. Importantly, the lymphopenia-inducing properties observed when fingolimod is administered orally (34) or i.v. were not observed when the drug was applied topically to the skin (see Supplemental Fig. 2).

It is well documented that fingolimod, once phosphorylated, is a modulator of the S1P receptors; however, a number of other targets for fingolimod have also been identified (52), including SK-1 (37, 53), ceramide kinase (54), 14-3-3 protein (55), acid sphingomyelinase (56), and phospholipase A₂ (57). To further support our hypothesis that inhibition of SK reduces histamine-induced inflammation, we examined two additional SK inhibitors: 1) MP-A08, a

FIGURE 2. Histamine-induced ear swelling is SK-1-dependent. (A) Fingolimod (10 μ g) or EPH was applied topically to the ears 24 h prior to histamine (8 mg) injection intradermally and measurement of ear thickness over time; *n* = 7 mice/group, in three individual experiments. **p* < 0.05 versus EPH + histamine. (B and C) Similar experiments were performed in *Sphk1*^{-/-} and *Sphk2*^{-/-} mice; *n* = 8 mice/group, in three individual experiments. **p* < 0.05 versus EPH + histamine. (D) Whole ears of WT mice were collected from (A) at 24 h after histamine administration for SK-1 and SK-2 activity assays. Data are expressed as means $\pm$ SEM for *n* = 8 mice/group in three individual experiments as arbitrary radioactive spot intensity units per milligram protein. **p* < 0.05 versus EPH + HMEM-PIPES, #*p* < 0.05 versus EPH + histamine. (E) VeraVec endothelial cells were pretreated with fingolimod (100 nM) or vehicle control for 1 h prior to 30 min incubation with [³H]S1P and histamine (25 μ M). Whole-cell lysates were collected for the measurement of S1P generated; *n* = 3 experiments. **p* < 0.05 versus control, #*p* < 0.05 versus histamine.



highly specific ATP-competitive inhibitor of SK1 and SK2 that is functionally distinct from fingolimod (41); and 2) the pan SK1/2 inhibitor [SKi (SKI-II) (40)]. As anticipated, when 10 μ g of either MP-A08 or SKi was applied topically to the ears 24 h prior to 8 mg histamine challenge, a significant reduction in histamine-induced ear swelling was observed over time (Fig. 1C and 1D, respectively).

To compare the role of SK-1 versus SK-2 in histamine-induced ear inflammation, we used the *Sphk1*^{-/-} and *Sphk2*^{-/-} mice and examined the effect of a prophylactic topical treatment of fingolimod. As shown in Fig. 2, when compared with the WT control animals (Fig. 2A), *Sphk1*^{-/-} mice responded poorly to 8 mg histamine for increased ear swelling (Fig. 2B) whereas the *Sphk2*^{-/-} mice exhibited an ear swelling similar to that observed in WT mice (Fig. 2C versus Fig. 2A). Of greater interest, both the WT and *Sphk2*^{-/-} mice responded to topical pretreatment of fingolimod with attenuated histamine-induced ear swelling (Fig. 2A, 2C) whereas the *Sphk1*^{-/-} mice exhibited no response. These results suggest a greater role for SK-1 than SK-2 in histamine-induced inflammation, and to investigate this further we used similar experiments in WT mice together with an enzymatic assay to determine SK-1 and SK-2 activity in whole-ear lysates. As shown in Fig. 2D, baseline SK-1 levels in mouse ears are greater than those of SK-2 and, as predicted from Fig. 2B and 2C, histamine significantly increased SK-1 but not SK-2 in this tissue (Fig. 2D). When 10 μ g fingolimod was topically applied to the mouse ear prior to 8 mg histamine for 6 h, SK-1 activity in the ear was reduced by ~50%. Of note, a similar response was observed with pretreatment of SKi, and no further reduction in SK-2 activity was observed with either inhibitor (Fig. 2D). Administration of SK inhibitors alone reduced baseline levels of SK-1 in two of the three mouse ears but this did not reach statistical significance, although two of the three mice tested showed a trend in this direction.

To determine whether administration of fingolimod also resulted in the reduction of S1P, we conducted similar experiments and examined whole-ear lysates for S1P production using a commercially available ELISA kit. Unfortunately, the ear-derived S1P from multiple mice fell below the level of reliable detection (data not shown). Determined to address this question, we turned to an in situ assay using the human VeraVec endothelial cells as the representative of an S1P-producing cell within the ear (30). Fig. 2E shows that within 30 min of exposure to 25 μ M histamine, S1P production is significantly increased. However, when pretreated with 100 nM fingolimod for 1 h, histamine is unable to produce S1P in these cells (Fig. 2E).

Epicutaneous application of fingolimod attenuates histamine-induced vasodilation, neutrophil recruitment, and proinflammatory mediators

Having focused on ear swelling 6–24 h after histamine exposure, we next wanted to determine whether prophylactic epicutaneous application of fingolimod also altered the early phase of inflammation, such as vasodilation. To do this we used similar experiments of histamine-induced mouse ear inflammation together with intravital microscopy and large molecular mass FITC-dextran (150 kDa) to investigate vascular leakage in the ear. First, we showed that in untreated mice, i.v. injection of FITC-dextran does not leak out of the ear vasculature during 15 min (Fig. 3). Next, following intradermal injection of 8 mg histamine, we observed a rapid increase in leakage of ear vascular fluid with 150 kDa FITC-dextran being released into the parenchymal tissue within 5 min of histamine administration (Fig. 3). In contrast, when treated 24 h prior with a topical application of fingolimod, histamine-induced extravasation of vascular fluid was significantly attenuated (Fig. 3).

Returning to later time points, we next investigated the cellular content of mouse ears in response to histamine for either 6 or 24 h without or with fingolimod pretreatment. Fig. 4A (left panel)

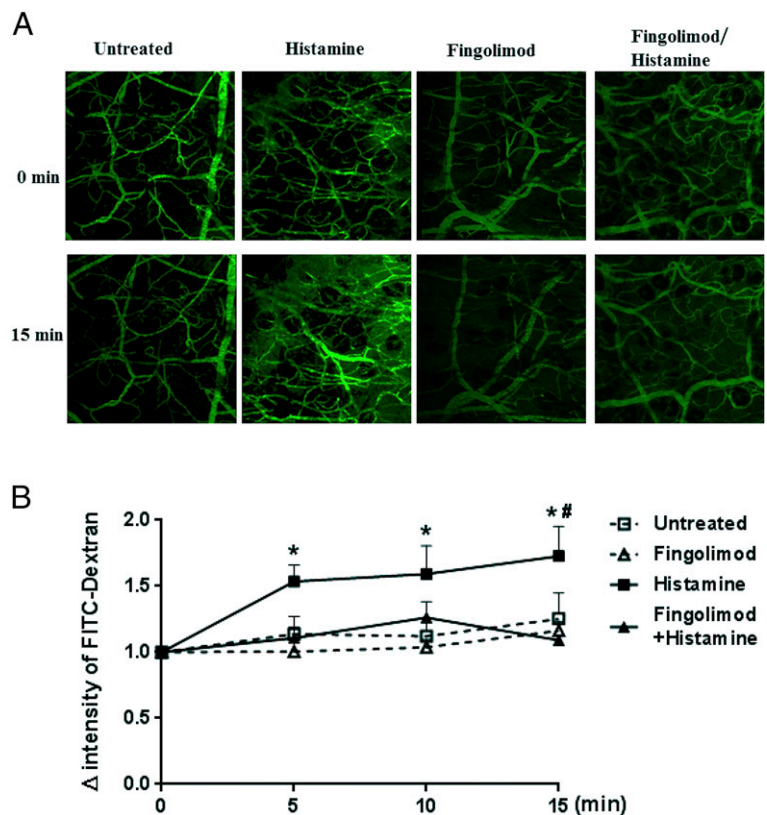


FIGURE 3. Epicutaneous application of fingolimod attenuates histamine-induced rapid vasodilation in vivo. **(A)** Fingolimod (10 μ g) or diluent (EPH) was applied to the ears of mice 24 h prior to FITC-dextran being injected retro-orbitally followed immediately by intradermal injection of 8 mg histamine into the same ear. Representative images from the ears of mice (untreated, histamine, fingolimod, fingolimod + histamine) at time 0 and 15 min after histamine injection and shown. Original magnification $\times 20$. **(B)** The fluorescence intensity of FITC was captured during 5-min intervals for up to 15 min, and pooled normalized data from $n = 4$ mice/group, in four individual experiments are shown. * $p < 0.05$ histamine versus untreated, # $p < 0.05$ fingolimod + histamine versus histamine.

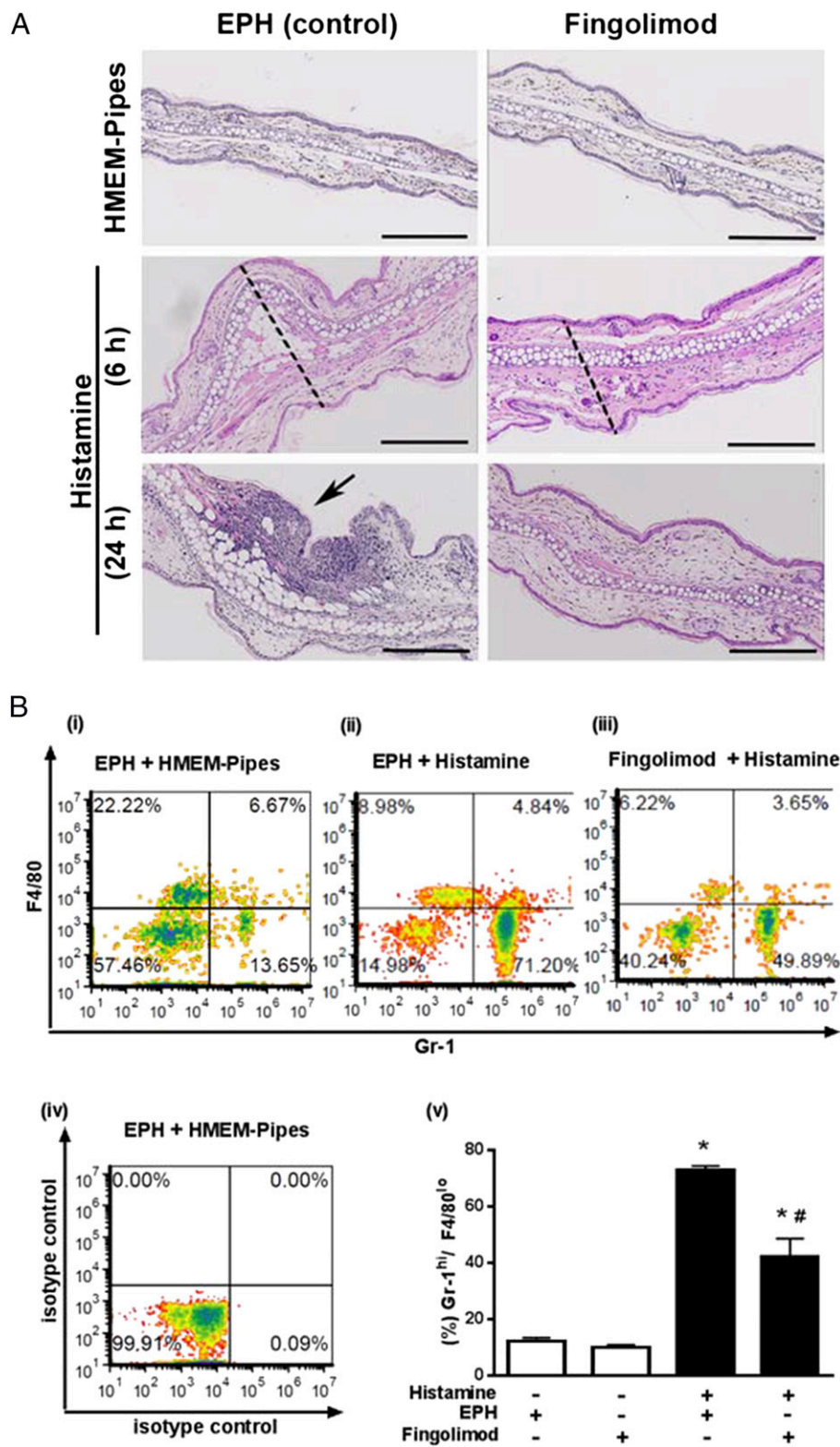
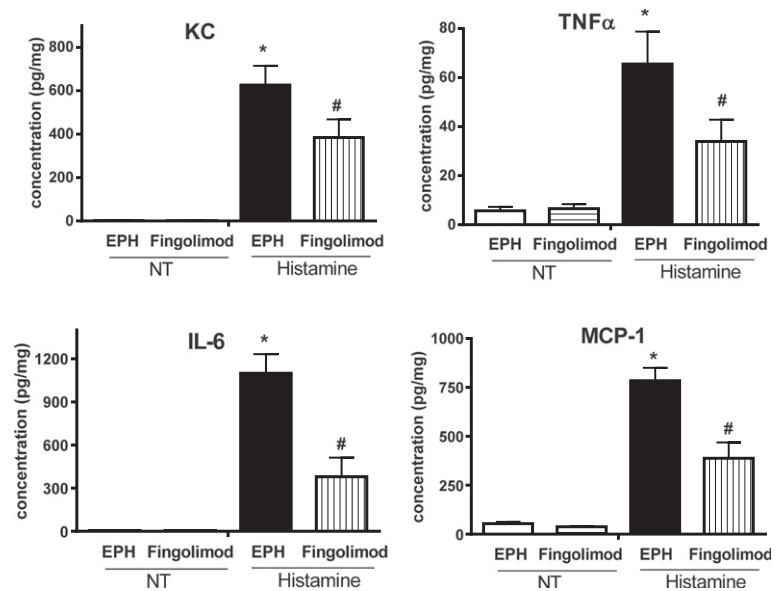


FIGURE 4. Epicutaneous application of fingolimod attenuates swelling and cellular infiltration to the ears. **(A)** H&E staining of fingolimod (10 μ g) or EPH-treated ears without or with histamine exposure for 6 or 24 h. Results are representative of $n = 9$ mice/group in 3 experiments at $\times 10$ original magnification, with the hashed line indicating ear thickness and an arrow illustrating the area of leukocyte infiltration. Scale bars, 300 μ m. **(B)** Fingolimod (10 μ g) or EPH (control) was applied topically to 8 mg histamine-treated ears, which were harvested after 24 h, digested, and immunostained for Gr-1 and F4/80 from **(i)** EPH-treated and untreated, **(ii)** EPH and histamine-treated, and **(iii)** fingolimod and histamine-treated ear lysates, showing **(iv)** isotype control Abs and **(v)** the quantified data; $n = 9$ mice/group, in three individual experiments. * $p < 0.05$ versus HMEM-PIPES, # $p < 0.05$ versus EPH + histamine.

confirms via H&E staining on the harvested and fixed ears that intradermal histamine challenge increases the ear thickness, likely via edema and vasodilation, within 6 h (ear diameter identified with a hashed line) and is followed by significant cellular infiltration at 24 h (identified with an arrow in Fig. 4A). Strikingly, prophylactic epicutaneous application of fingolimod perturbed both histamine-induced vasodilation and cellular infiltration (Fig. 4A, right panel) and was evident in all nine mice examined per group.

To investigate whether Gr-1^{hi}/F4/80^{lo} granulocytes were recruited, flow cytometric analysis was performed on single-cell suspensions from ear digests 24 h after histamine injection. Using a forward scatter and side scatter gating strategy, coupled with the markers Gr-1 and F4/80, the granulocyte populations of interest were investigated. The Gr-1^{hi}/F4/80^{lo} granulocytes within the ears of control mice constituted ~15% when compared with isotype controls (Fig. 4Bi, 4Bv). When treated with histamine ~70% of the gated cells were Gr-1^{hi}/F4/80^{lo} (Fig. 4Bii, 4Bv). Prophylactic

FIGURE 5. Epicutaneous application of fingolimod attenuates histamine-induced proinflammatory cytokine production in mouse ears. Fingolimod (10 μ g) or EPH was applied topically to ears, and following 24 h exposure to 8 mg histamine, ears were harvested, digested, and cytokines were detected via microbead array and flow cytometric analysis. Quantified results are normalized to BSA (total protein) in each lysate for KC, TNF- α , MCP-1, and IL-6; $n = 7$ –9 mice/group, in three individual experiments. * $p < 0.05$ versus EPH/NT, # $p < 0.05$ versus EPH/histamine.



epicutaneous treatment of fingolimod attenuated the histamine-induced Gr-1^{hi}/F4/80⁺ granulocyte population (Fig. 4Biii, v). Of note, the circulating granulocyte numbers did not change, suggesting that epicutaneous treatment of fingolimod does not alter their mobilization from bone marrow and secondary lymphoid organs (see Supplemental Fig. 3).

As murine KC is a potent chemoattractant for neutrophils (58) and histamine stimulates KC production (59), we next examined whether KC levels were modified in response to fingolimod application. As shown in Fig. 5, 24 h after histamine injection levels of KC were significantly elevated within the ears of the mice. Prophylactic epicutaneous treatment with fingolimod significantly attenuated histamine-induced KC production. Similarly, fingolimod reduced TNF- α , MCP-1, and IL-6 levels in the mouse ears (Fig. 5). No histamine-induced increases in IL-12p70, IFN- γ , and IL-10 levels were detected (data not shown).

Established skin inflammation is rapidly resolved by applying fingolimod

With data suggesting that epicutaneous application of fingolimod prior to histamine exposure can attenuate inflammation in the ear, we next examined whether this drug could be used as a treatment for established inflammation. Returning to the aforementioned mouse model of histamine-induced ear skin inflammation we observed that ear swelling was also rapidly resolved when fingolimod was applied epicutaneously 30 min after histamine injection (Fig. 6A).

To evaluate the extent by which fingolimod can attenuate established immune responses we tested it in an IgE-dependent passive cutaneous anaphylaxis (PCA) model (42). This PCA reaction develops with infiltrating leukocytes (predominantly neutrophils) via mast cell activation, degranulation, and release of proinflammatory mediators such as histamine (42, 60). As shown in Fig. 6B, ear swelling was observed within 15 min after DNP-HSA injection in IgE-sensitized ear pinnae, which peaked at 30 min and gradually subsided during 6 h. When applied topically to the ears 30 min after DNP-HSA injection, fingolimod significantly reduced ear swelling within 90 min (Fig. 6B).

Epicutaneous application of fingolimod prevents histamine-induced neutrophil recruitment

To determine whether epicutaneous application of fingolimod specifically blocks histamine-induced leukocyte rolling and

adhesion in the ear, intravital microscopy was performed with i.v. retro-orbital injection of rhodamine 6G used to visualize circulating leukocytes (61). As shown in Fig. 7A and Supplemental Video 1, intradermal injection of histamine into the ear significantly increased leukocyte rolling in the postcapillary venules

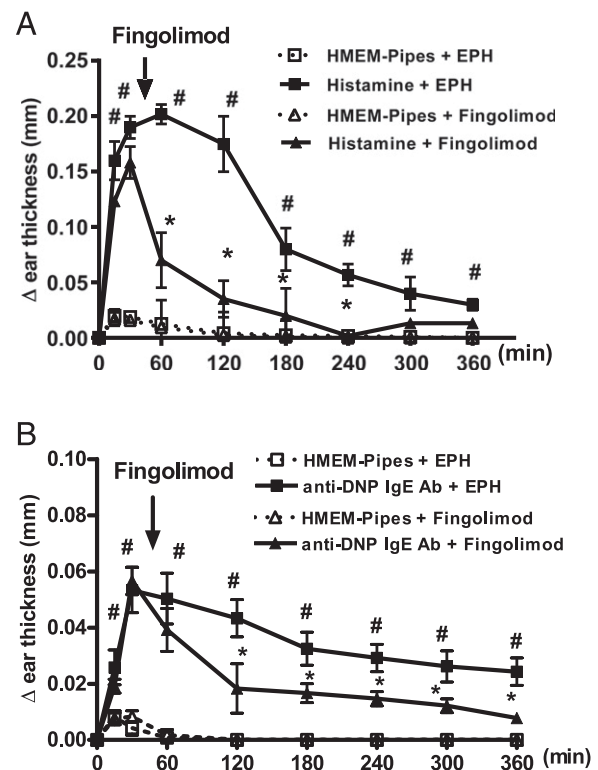


FIGURE 6. Epicutaneous application of fingolimod attenuates histamine- and PCA-induced ear swelling. (A) Fingolimod (10 μ g) or EPH (control) was applied topically to both ears at 30 min after histamine (8 mg) and HMEM-PIPES (control) intradermal injections. Ear thickness was measured over time; $n = 9$ mice/group, in three individual experiments. # $p < 0.05$ versus HMEM-PIPES + EPH, * $p < 0.05$ versus histamine + EPH. (B) To the ears of anti-DNP IgE-sensitized mice (or HMEM-PIPES controls), DNP-HSA was injected i.v. followed 30 min later by fingolimod (10 μ g) or EPH topical application. Ear thickness was measured; $n = 9$ mice/group, in three individual experiments. # $p < 0.05$ versus HMEM-PIPES + EPH, * $p < 0.05$ versus anti-DNP IgE Ab + EPH.

within 1 h and remained elevated for at least 24 h. At 30 min after histamine challenge, epicutaneous application of fingolimod significantly attenuated leukocyte rolling within 1 h of histamine exposure and was sustained for at least 24 h. A similar response was observed for leukocyte adhesion in the ear vasculature (Fig. 7B, Supplemental Video 1). In contrast, epicutaneous application of histamine receptor antagonists (0.5% chlorpheniramine and 0.5% cimetidine for H1 and H2, respectively) failed to reduce leukocyte rolling and adhesive events (Fig. 7B, Supplemental Video 1).

In humans, epicutaneous application of fingolimod perturbs histamine- and allergen-induced inflammation

Finally, we tested the effect of fingolimod in a human study wherein healthy participants underwent a routine SPT into the inner forearm skin, the primary mode of skin testing for immediate IgE-mediated allergic reactions against *Alternaria* (mold), *D. pteronyssinus* (dust mite), cat dander, and rye grass (46). Details of the participant inclusion/exclusion criteria are listed in *Materials and Methods*, with our protocol being that at 10 and 20 min after SPT, fingolimod (0.0125 mg; extrapolated from the mouse data) was applied topically at the skin prick site of histamine and the most reactive allergen on one forearm whereas the other forearm received the diluent (EPH) only to the same test sites. Overall, we observed that fingolimod significantly, albeit modestly, reduced the histamine-induced wheal response. The kinetics of a histamine-induced wheal for a representative participant is shown in Fig. 8A with the collective data of all participants represented in Fig. 8C. No significant reduction in histamine-induced flare was observed when fingolimod was applied (Fig. 8E). A more pronounced effect was observed in the allergen-induced SPT with a positive response to fingolimod treatment by a representative participant following a rye grass skin prick (Fig. 8B) and the pooled data of all participants (Fig. 8D). A significant reduction in allergen-induced flare was also observed when fingolimod was applied and is represented as pooled data in Fig. 8F. Based on predetermined human clinical safety data for fingolimod, we monitored heart rate, blood pressure, and circulating leukocyte counts before and after the experimental procedure. Repeated electrocardiograms and blood

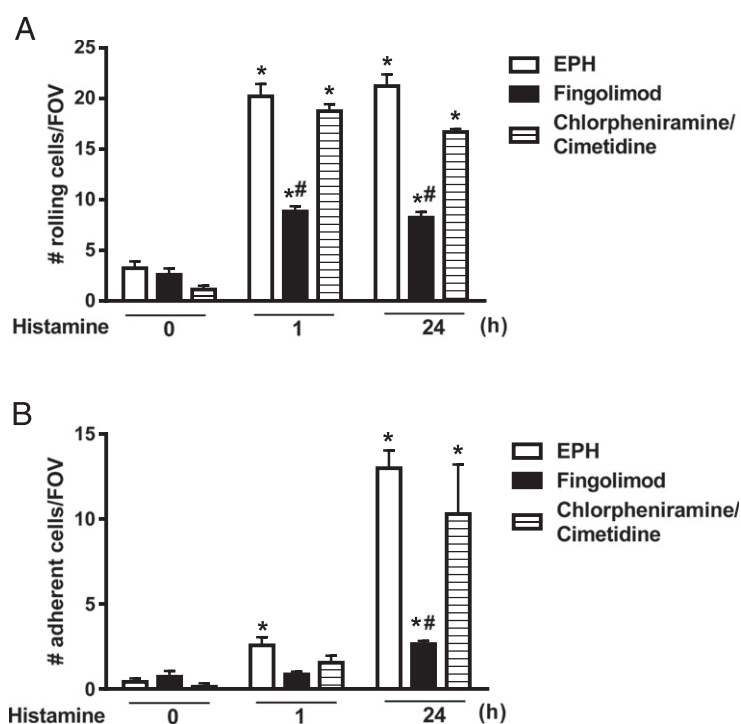
tests confirmed that no adverse events occurred in response to topical application of fingolimod.

Discussion

This study reveals a novel therapeutic approach to treat skin inflammation via topical application of fingolimod. By inhibiting the SK-1/S1P axis, histamine- and PCA-induced neutrophil recruitment is attenuated in the ear of mice. Moreover, fingolimod is equally effective when applied both before and after histamine/allergen challenge, with significant reductions observed in the production of localized chemoattractive cytokines KC, TNF- α , IL-6, and MCP-1. Translation into a human study supported our animal work with histamine- and allergen-induced SPTs significantly perturbed when fingolimod was topically applied to the established inflammatory response.

Fingolimod is a sphingosine analog that is taken up by cells, phosphorylated by SK2 (34), and is released as fingolimod-P, which then binds to and activates S1P_{1,3-5} but not S1P₂ (35, 62). Fingolimod-P also induces a later “functional antagonism” by promoting the polyubiquitination, endocytosis, and proteosomal degradation of S1P₁, which creates S1P₁-null lymphocytes, resulting in inhibition of lymphocyte egress from lymph nodes and thymus, thus causing lymphopenia (35, 63–65). Because of these attributes, fingolimod is indicated to treat multiple sclerosis at high daily oral doses (0.5–3.5 mg). Fingolimod is also effective in vivo against other pathologies when administered via alternate routes. For example, intranasal administration of fingolimod at 6 μ g/day for 9 d significantly attenuated OVA-induced recruitment of eosinophils and dendritic cells (DCs) in the nasal mucosa of mice with allergic rhinitis without altering circulating lymphocyte counts (66). Topical prophylactic application of fingolimod to mice with allergic contact dermatitis at 10 μ g/day for 9 d also inhibited DC migration and reduced the circulating lymphocyte counts (67). Systemic administration of fingolimod showed a dose-dependent effect on aggravated ventilator-induced lung injury in mice, with 2 μ g enhancing endothelial barrier function and reducing lung permeability, and 4 μ g causing endothelial apoptosis and increasing lung permeability (68). Our own work

FIGURE 7. Epicutaneous application of fingolimod attenuates histamine-induced cell rolling and adhesion. Mice were injected intradermally with histamine (8 mg) followed at 30 min with topical treatment of fingolimod (10 μ g) or chlorpheniramine and cimetidine (0.2 mg). (A) Rolling and (B) adherent leukocytes were observed in postcapillary venules of the ears using intravital microscopy at 0, 1, and 24 h after histamine injection; $n = 6$ –8 mice/group, in three individual experiments. * $p < 0.05$ versus corresponding 0 h of EPH/fingolimod/chlorpheniramine + cimetidine treatment, # $p < 0.05$ versus histamine + EPH at corresponding time points.



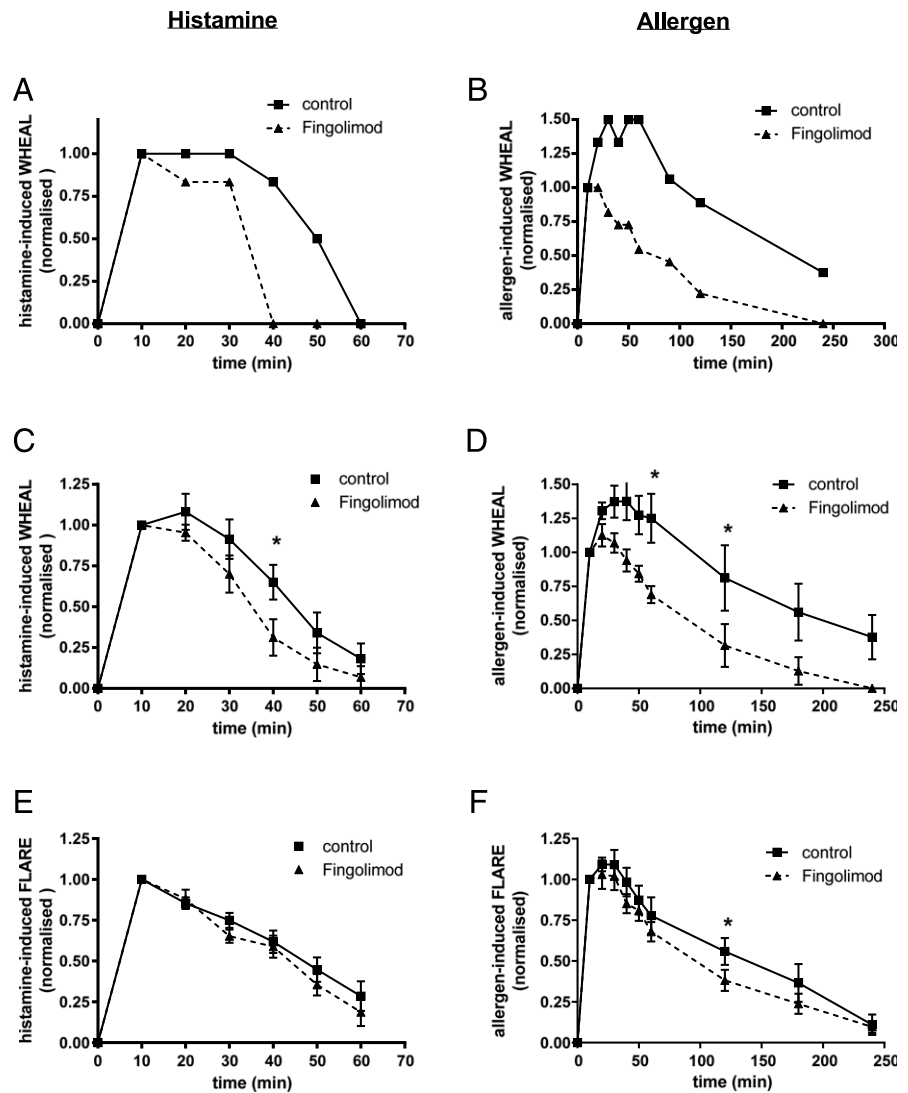


FIGURE 8. Epicutaneous application of fingolimod attenuates histamine- and allergen-induced human skin inflammation. Healthy participants underwent routine SPTs. **(A)** Example of normalized histamine-induced wheal response. **(B)** Example of normalized allergen-induced wheal response. Pooled data from wheal responses for **(C)** histamine and **(D)** allergens, as well as pooled data for flare responses to **(E)** histamine and **(F)** allergen are shown as means $\pm$ SEM from $n = 13$ histamine and $n = 8$ allergens in six individual experiments. * $p < 0.05$ using a two-way ANOVA with the Bonferroni posttest for repeated measures and multiple comparisons versus corresponding controls.

demonstrated that a single i.p. injection of 10 μ g fingolimod for just 1 h attenuated histamine-induced neutrophil recruitment to the mouse cremaster muscle (29). This was corroborated by a more recent study showing that S1P₃, G α q, phospholipase C β , and Ca²⁺ are employed to mobilize P-selectin in response to histamine (69). The conceptual advance in this study is the discovery that epicutaneous application of fingolimod on the skin of mice (10 μ g) and humans (25 μ g) significantly perturbs established inflammation. Importantly, our ability to rapidly attenuate established inflammation in either mouse or human skin (Figs. 6, 8) provides some insight into the mode of action of this drug. Based on our observations, we suggest that fingolimod acts independent of 1) SK-2-mediated phosphorylation, 2) internalization of the S1P receptors, and 3) degradation of SK. The precise mechanisms underpinning its effect are still to be determined; however, with documentation that fingolimod inhibits other signal transduction pathways such as SK-1 (37, 53), ceramide kinase (54), 14-3-3 protein (55), acid sphingomyelinase (56), and phospholipase A₂ (57), efforts to identify them and then chemically modify the drug to generate clinically relevant analogs is warranted. To this end, Chen et al. (70) recently described azacyclic fingolimod analogs that fail to activate S1P₁ and S1P₃ receptors in vivo (such that the lymphopenia- and bradycardia-inducing properties are avoided) but retain inhibitory effects on nutrient transporter proteins and anticancer activity in solid tumor xenograft models. Thus, designing

analogues for absorption into the skin to treat allergic inflammation is potentially possible and deserves further investigation.

Our epicutaneous application of fingolimod (0.025 mg) onto human skin was well tolerated and demonstrated significant reduction in histamine- and allergen-induced SPT. Notably, the effect on the established wheal was more pronounced than that on the flare and is likely due to the variability in obtaining these measurements from participants with differences in skin pigmentation. Of note, our participants were from different ethnicities, thus constituting a broad range of skin phototypes where 7 of the 13 participants exhibited skin type II, 3 of 13 type III, 2 of 13 type IV (Asian), and 1 of 13 type V (Indian) (71). Also, the concentration of fingolimod applied to the skin was 20-fold lower than that shown to cause lymphopenia and transient bradycardia in humans when administered orally (49). Our repeated electrocardiograms and blood tests confirmed that neither of these occurred. Topical application of fingolimod to mouse skin showed reduced vascular permeability, neutrophil recruitment, ear swelling, as well as attenuated production of proinflammatory cytokines. The reduction in neutrophils may be clinically important as neutrophils isolated from allergic patients release 5-fold more histamine upon allergen challenge when compared with neutrophils from healthy donors (11). Fingolimod may also be acting on dermal mast cells, circulating basophils and eosinophils, Langerhans cells, and resident DCs based on their proximity to the drug when it is administered.

This will also lead to changes in the local production of leukotrienes, cytokines (e.g., IL-1 β , IL-10 and IL-22), and chemokines important for developing the allergic response (reviewed in Ref. 72) and thus remains to be determined in future studies.

A precedent for using small molecules in topical treatments to reduce allergic contact inflammation in rodents includes prophylactic application of dasatinib and LCB 03-0110 (inhibitors for tyrosine kinases) (73), small interfering RNA against CD86 (an inhibitor of DC migration) (74), and PAP-1 (voltage-gated potassium channel inhibitor targeting CD8 T cell infiltration) (75). Prophylactic topical treatment with antihistamines enhances barrier permeability function but this effect is short-lived and lost within 2 h (76). Price et al. (38) have shown that repeated intranasal treatment with the SK-1 inhibitor SK-1-I can suppress mast cell activation and proinflammatory cytokine production via an NF- κ B-dependent pathway in OVA-induced asthmatic mice. Our present study reveals a new therapeutic opportunity for fingolimod, as we demonstrate that topical application of this drug is effective both before and after inflammatory reaction with an effect that is maintained beyond 24 h. More exciting is that with a half-life of $\sim$ 9 d (77), the therapeutic potential of fingolimod to sustain an anti-inflammatory microenvironment extends well beyond the experimental limitations shown in this study.

Inflammation is a growing global health issue with $\sim$ 30–40% of the world's population currently suffering from one or more allergic conditions. Complex allergies involving polysensitization and multiple organ involvement are also on the increase, with a high morbidity placing a higher demand on health care delivery services. It is also forecast that allergy-associated pathologies will increase further as air pollution and the ambient temperature increases (78). Clearly, new therapeutic approaches are desperately needed. This study has shown that SK-1 regulates skin inflammation and that fingolimod could be repurposed as a new treatment option and applied topically (before or after allergen exposure) to significantly attenuate the neutrophilic inflammatory response.

Acknowledgments

We thank Zelig Eshar (Weizmann Institute of Science, Rehovot, Israel) for providing IgE anti-DNP mAb-producing mouse SPE-7 hybridoma cells and Dave Yip and Tim Hercus for IgE anti-DNP mAb purification.

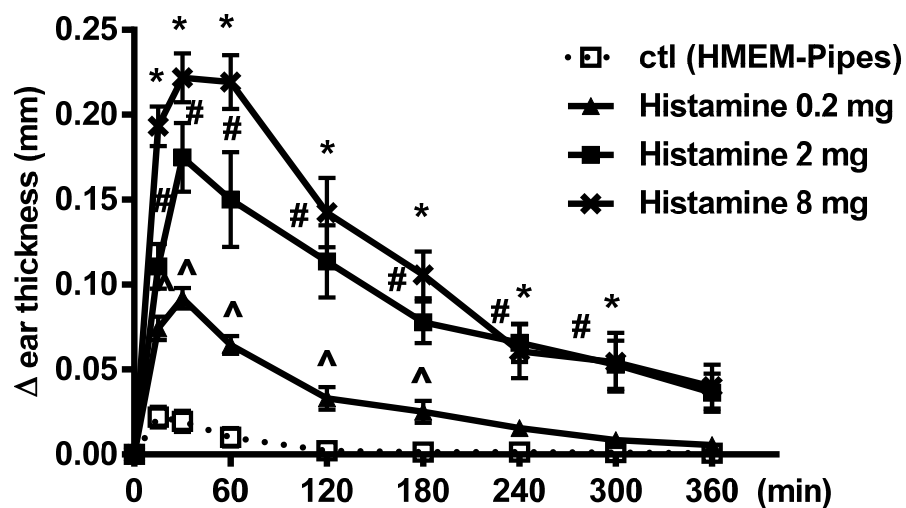
Disclosures

The authors have no financial conflicts of interest.

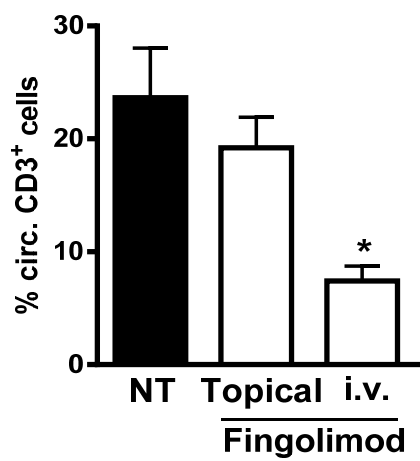
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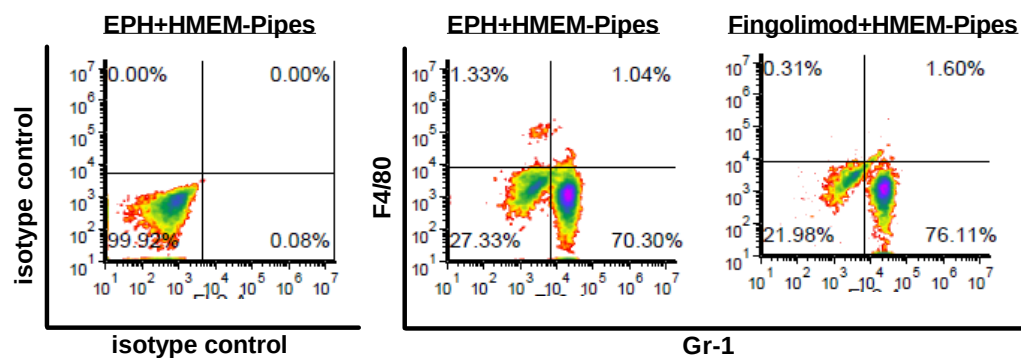
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Supplemental Figure 1. Histamine-induced inflammation of the ear is dose dependent. Mice were injected intradermally with HMEM-Pipes vehicle (control, left ear) or various physiologically relevant concentrations of histamine (right ear). Ear thickness was measured at 0, 15, 30, 60, 120, 180, 240, 300 and 360 min post-histamine injection. $n=8-10$, in 3-5 individual experiments, *, #, ^, $p<0.01$ vs ctl.



Supplemental Figure 2. Epicutaneous application of Fingolimod does not induce lymphopenia. Fingolimod (10 μ g) was delivered either topically to the ears or i.v. injection (retro-orbital) 24 h prior to blood being drawn, red blood cell lysis and immuno-staining for CD3, n=9, in 3 individual experiments.



Supplemental Figure 3. Epicutaneous application of Fingolimod does not reduce the mobilization of neutrophils. Fingolimod or vehicle control (EPH) was applied topically 24 h following HMEM-Pipes vehicle intradermal injection to the ear pinna of mice. Blood was drawn at 48 h post-Fingolimod or EPH application prior to red blood cell lysis and immuno-staining for Gr-1 and F4/80. A representative figure of the granulocyte gated cells from n=6 in 3 individual experiments.

Supplemental Data

Supplemental video S1. Intravital videos from untreated and histamine treated (8 mg, 30 min) mice following anti-histamines (Chlorpheniramine & Cimetidine; both at 200 µg) or Fingolimod (10µg) topical treatment to the ear. Videos are 2 sec images.