The Sphingosine-1-Phosphate Receptor Modulator Fingolimod Aggravates Murine Epidermolysis Bullosa Acquisita


TO THE EDITOR

Fingolimod (FTY720) is the first orally available disease-modifying drug against relapsing-remitting multiple sclerosis (MS) and the first drug of the class of sphingosine-1-phosphate receptor modulators (S1PRMs). Sphingosine-1-phosphate receptors are extracellular, G protein-coupled receptors regulating diverse cell responses, including cell migration and lymphocyte egress from the lymph nodes. Fingolimod is a functional antagonist of sphingosine-1-phosphate receptor 2 and sphingosine-1-phosphate receptor 3–5 (Thieme et al., 2017).

In addition to MS, fingolimod suppresses disease in models of several chronic inflammatory diseases including psoriasis, rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease (IBD), and dermatomyositis. It also exerts therapeutic, neuroprotective effects in mouse models of neurodegenerative diseases, including Parkinson and Alzheimer disease (Aytan et al., 2016; Zhao et al., 2017). Accordingly, fingolimod and other S1PRMs are currently investigated for several indications, including IBD and psoriasis, in clinical trials (Chew et al., 2016; Mao-Draayer et al., 2017; Park and Im, 2017).

In contrast, the therapeutic effectiveness of S1PRMs on pemphigoid diseases, a group of prototypical and organ-specific autoimmune diseases of the skin and mucous membranes, has not been evaluated. Epidermolysis bullosa acquisita (EBA) is a pemphigoid disease characterized by an autoantibody-driven immune response against type VII collagen (Kasperkiewicz et al., 2016). The pathogenesis of EBA is still largely elusive, but the emergence of the disease is associated with IBD (Reddy et al., 2013). With S1PRMs effective in a wide spectrum of other autoimmune diseases, including IBD, they may also be most valuable in the treatment of EBA, particularly when IBD is concomitant.

We have therefore examined the effect of fingolimod on disease in the antibody transfer EBA and in the immunization-induced EBA mouse model (Kasperkiewicz et al., 2016). To address the therapeutic potential of fingolimod in the immunization-induced EBA mouse model, mice were immunized against the von-Willebrand-factor A-like domain 2 of type VII collagen and were randomly assigned to the fingolimod or the vehicle control group, when >2% of their total body surface were affected by skin lesions. Afterwards, disease severity under this treatment was monitored for 5 weeks. By week 2, fingolimod-treated mice exhibited significantly more severe disease than vehicle-treated mice (Figure 1a and b). Lesional skin of both fingolimod- and vehicle-treated mice exhibited histopathologic changes typical for EBA, with dense dermal infiltrates of neutrophils and subepidermal clefts (Figure 1c). To scrutinize whether fingolimod may affect autoantibody production, we assayed serum titers of anti-COL7 IgG autoantibodies. Fingolimod treatment did not alter levels of circulating anti-COL7 IgG autoantibodies (Figure 1d). This suggests that the difference between the two groups is due not to an effect of fingolimod on autoantibody production, but to a direct impact of fingolimod on the effector phase. In line with this conclusion, fingolimod also aggravated skin inflammation in the antibody transfer EBA mouse model (Figure 1e), which exclusively reflects the effector phase (Sadik et al., 2018).

The effector phase of EBA in our mouse models is differentially

Abbreviations: BP, bullous pemphigoid; EBA, epidermolysis bullosa acquisita; IBD, inflammatory bowel disease; MS, multiple sclerosis; S1PRMs, sphingosine-1-phosphate receptor modulators; Treg, regulatory T cell

Accepted manuscript published online 7 June 2019; corrected proof published online 16 August 2019 © 2019 The Authors. Published by Elsevier, Inc. on behalf of the Society for Investigative Dermatology.
modulated by T cells through the local interaction of neutrophils with γδ T cells and natural killer T cells promoting disease, and regulatory T cells (Tregs) counteracting it (Bieber et al., 2017; Bieber et al., 2016). We therefore determined the mRNA levels of T-cell markers and prototypical T cell–derived cytokines in relation to the density of neutrophils in the dermal infiltrate, which was assessed by the mRNA expression level of the neutrophil cell surface marker Ly-6G (Figure 2a). While the expression levels of the pan-T-cell surface marker CD3 and those of TCR-γ and TCR-β only tended toward reduced levels in lesional skin of fingolimod-treated mice compared with vehicle-treated mice, mRNA levels of FoxP3, the signature transcription factor of Tregs, were significantly decreased. Similarly, when determined on the protein level, the FoxP3+ cells tended to be decreased in fingolimod-treated mice relative to the degree of neutrophil infiltration (Figure 2b). Additionally, this was subsequently confirmed for lesional skin in the antibody transfer model (Supplementary Figure S1 online).

Profiling the mRNA expression levels of cytokines in lesional skin, we found that those of IL-10 were decreased in fingolimod-treated mice, while IL-17A and IFN-γ levels did not differ from those in vehicle-treated mice (Figure 2c). IL-10 is a major effector molecule of Tregs and can counteract the effector phase of pemphigoid diseases by paracrine actions on neutrophils (Kulkarni et al., 2016). Therefore, its reduction in the skin is possibly a mechanism contributing to the aggravation of skin inflammation upon fingolimod treatment.

We also assessed the relative percentage of Tregs among the T-cell populations in the draining lymph nodes of inflamed skin. Fingolimod treatment significantly enhanced the percentage of Tregs in the lymph nodes (Figure 2d). Thus, our results collectively suggest that one mechanism of fingolimod-driven exacerbation of EBA may be an impairment of the migration of a sufficient number of Tregs from the lymph nodes into the skin.

These unexpected findings suggest that fingolimod may promote skin inflammation in patients with EBA. Therefore, special attention will also be required when administering fingolimod or other S1PRMs to patients with IBD, because these are considered to be, in general, already prone to the development of EBA.
As the different pemphigoid diseases share several common features, the results of our study on the effect of fingolimod on EBA warrant future studies to address the effect of S1PRMs on models of bullous pemphigoid (BP). BP is the most common pemphigoid disease and is closely associated to MS and several neurodegenerative diseases, such as Parkinson and Alzheimer disease (Bastuji-Garin et al., 2011; Kibsgaard et al., 2017; Langan et al., 2011). The use of S1PRMs may precipitate or aggravate BP in these patients. Thus, future studies must scrutinize whether the administration of fingolimod (e.g., to patients with MS) further enhances these patients’ risk to develop BP. Respective data are not available yet because fingolimod was licensed for use in MS only in 2010, and the onset of BP following that of MS happens on average more than a decade later (Kibsgaard et al., 2017).

Data availability statement
All primary datasets generated for this study are available from the corresponding author upon request.

Ethics statement
All mouse experiments were approved by the state government of Schleswig-Holstein.

CONFLICT OF INTEREST
The authors state no conflict of interest.

ACKNOWLEDGMENTS
This work is supported by Deutsche Forschungsgemeinschaft (DFG), grant no. Sa1960/5-1.

AUTHOR CONTRIBUTIONS
Conceptualization: CDS; Formal Analysis: YG; Investigation: MT, KB, TS, MW, SM; Methodology: CDS, KK; Visualization: CDS; Writing - Original
Clarifying the Current Understanding of Syndromic Basal Cell Carcinomas

TO THE EDITOR

Chiang et al. presented important data on the lower mutational load and increased genomic stability of basal cell nevus syndrome (BCNS)—basal cell carcinomas (BCCs) in contrast to sporadic BCCs in an original article titled “Genomic Stability in Syndromic Basal Cell Carcinoma” (Chiang et al., 2018).

Although they concluded that BCNS-BCCs show better therapeutic response than sporadic BCCs to Smoothened inhibitors (SIs) because of lower mutational load, it is important to note that some patients with BCNS do not carry PTCH1 mutations and therefore may not respond to SIs. In fact, we previously reported that 10–12% of genetically tested patients with BCNS failed to have chromosome 9/PTCH1 mutations despite meeting the clinical criteria for BCNS (Shih et al., 2018). Moreover, only 28.5% of 288 patients with BCNS received genetic testing for chromosome 9/PTCH1 mutations, though the types of mutations were not specified (Shih et al., 2018). We want to highlight the importance of genetic testing before initiating SI therapy, because patients with BCNS with germline mutations in non-PTCH1 genes, such as SUFU, are unlikely to respond to SIs (Smith et al., 2014; Varjosalo and Taipale, 2008). There has also been a report of segmental BCNS caused by postzygotic mutation in SMO, which would render SI therapy ineffective (Khamaysi et al., 2016).

Additionally, several of the BCNS-BCCs sequenced in Chiang and colleagues’ study did not express any of the mutations they tested for (BCNS6, BCNS8, BCNS12, BCNS14, and BCNS15), leaving a gap in the understanding of the drivers behind those tumors (Chiang et al., 2018).

We propose that future studies be designed to further enhance the understanding of genetic drivers behind BCNS. Chiang et al. obtained whole-exome analysis of 20 BCCs from 16 patients with BCNS. BCNS-BCCs in the study were found to harbor fewer UV signature mutations than sporadic BCCs. In future studies, obtaining sequencing data from multiple lesions
SUPPLEMENTARY MATERIALS AND METHODS

Mice

C57BL/6j wild-type mice were purchased from Charles River Laboratories (Sulzfeld, Germany). B6.SJL-H2s C3c/Cyj wild-type mice were bred in the animal facility of the University of Lübeck (Lübeck, Germany). All animals were housed in a 12-hour light-dark cycle in the animal facility of the University of Lübeck. All experiments were performed in 8- to 12-week-old age- and sex-matched mice by certified personnel. All experiments had been permitted in advance by the Schleswig-Holstein state government.

Generation of anti-Col7 IgG and recombinant von-Willebrandt-Factor A-like domain 2 (vWF2A)

To generate anti-Col7 IgG, rabbits were immunized against three epitopes of type VII collagen, and IgG directed to the epitope C ("anti-COL7 IgG") was subsequently isolated from rabbit serum, as previously described (Sitaru et al., 2005). Purified anti-Col7 IgG was filter-sterilized (pore size 0.2 μm), and its concentration was determined by NanoDrop (ThermoFisher Scientific, Waltham, MA). Before use, the reactivity of each batch of anti-Col7 IgG was checked on murine tail skin sections by indirect immunofluorescence microscopy. Recombinant vWF2A was expressed and purified, as previously described (Bieber et al., 2017; Iwata et al., 2013). Described (Bieber et al., 2017; Iwata et al., 2013). To score the severity of disease in both models, skin areas exhibiting erythema, blisters, erosions, crusts, or alopecia were categorized as "affected" (Sitaru et al., 2005). Then, the percentage of the total body surface affected by skin lesions was calculated. In the antibody transfer model, mice were scored every other day starting at day 0. In the immunization-induced model, mice were scored weekly starting 4 weeks after the injection of vWF2A protein, as previously described (Bieber et al., 2017; Iwata et al., 2013).

Fingolimod administration

Fingolimod (FTY720) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Fingolimod was dissolved in 0.9% NaCl, and 5 mg per kg body weight were injected daily intraperitoneally starting on day 2 in antibody transfer EBA. In immunization-induced EBA, the administration of fingolimod started when the disease score reached ≥2% in individual mice.

FACS analysis of regulatory T cells (Tregs)

Cells were isolated from lymph nodes and the spleen by mincing the tissue on a 70-μm cell strainer (Corning, Corning, NY) and rinsing the tissue with 1% BSA in phosphate buffered saline (PBS). Spleen suspensions underwent erythrocyte lysis with hypotonic PBS (1:5 v/v). Resin suspensions were adjusted to 10^7 cells/ml. Afterward, cells were fixated using TrueNuclear Transcription Factor Buffer Set (BioLegend, San Diego, CA) and an Alexa647-conjugated anti-FoxP3 (BD Bioscience, San Jose, CA) antibody. Eventually, cells were stained using trypan blue (Merck KGaA, Darmstadt, Germany) and a Neubauer counting chamber. Cell suspensions then were adjusted to 10^6 cells/ml. After adjusting the cell number, unspecific binding was blocked using Fcγ receptor blocking solution (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Then, cells were stained with a FITC-conjugated anti-CD4 (Miltenyi) and a phycoerythrin-conjugated anti-CD25 (Miltenyi). To stain for FoxP3, cells were fixated using True-Nuclear Transcription Factor Buffer Set (BioLegend, San Diego, CA) and an Alexa647-conjugated anti-FoxP3 (BD Bioscience, San Jose, CA) antibody. After staining, cells were counted with the Miltenyi MACSQuant Analyzer 10. Results were analyzed using FlowJo (BD Bioscience, San Jose, CA).

Determination of anti-vWF2A IgG titers

Blood was collected by heart puncture, and serum was harvested and stored at −20°C until usage. Titers of antibodies directed to vWF2A of type VII collagen were determined using a modified version of a previously published protocol (Iwata et al., 2013). Briefly, an ELISA high binding 96-well plate (Corning, Corning, NY) was coated with recombinant 2.5 μg/ml vWF2A protein in 0.05 M sodium carbonate-bicarbonate coating buffer (pH 9.6) over night at 4°C. Afterwards, the plate was washed with PBST (PBS + 0.5% Tween20), and unspecific binding was blocked using 1% BSA (biotin-free) in PBST for 30 minutes. Then, the plate was washed again before sera were added in three dilutions (1:1000, 1:4000, 1:16000) and incubated for 1 hour. After another washing step, the detection antibody mouse IgG ELISA Quantitation Set (Bethyl, Montgomery, TX) was added. After washing with PBST, 50 μl TMB was added and color reaction was stopped with 0.2 M sulfuric acid. Optical densities were measured using the ELISA reader at 450 nm (Thermo Fischer Scientific).

Histopathology and immunofluorescence staining

For histopathology, biopsies of lesional skin were fixed in 4% Histofix solution (Carl Roth, Karlsruhe, Germany) and embedded in paraffin. From these, 6-μm paraffin sections were cut and hematoxylin and eosin stained. For immunofluorescence staining, lesional skin biopsies were embedded in TissueTek Cryomold (VWR, Darmstadt, Germany) before 6-μm sections were cut and stored at −20°C until usage. Biotinylated rat anti-mouse Ly6G Ab (BioLegend, San Diego, CA) in combination with DyLight 488-conjugated streptavidin (ThermoFisher Scientific) was used to detect Ly6G+ cells. Eventually, slides were mounted with DAPI fluorescent mounting medium (SouthernBiotech, Melbourne, Australia), visualized, and photographed using the BZ-9000 series Keyence microscope (Keyence GmbH, Neu-Isenburg, Germany), and www.jidonline.org 2384.e1

M Thieme et al.
Fingolimod Aggravates EBA

Histopathology and immunofluorescence staining

For histopathology, biopsies of lesional skin were fixed in 4% Histofix solution (Carl Roth, Karlsruhe, Germany) and embedded in paraffin. From these, 6-μm paraffin sections were cut and hematoxylin and eosin stained. For immunofluorescence staining, lesional skin biopsies were embedded in TissueTek Cryomold (VWR, Darmstadt, Germany) before 6-μm sections were cut and stored at −20°C until usage. Biotinylated rat anti-mouse Ly6G Ab (BioLegend, San Diego, CA) in combination with DyLight 488-conjugated streptavidin (ThermoFisher Scientific) was used to detect Ly6G+ cells. Eventually, slides were mounted with DAPI fluorescent mounting medium (SouthernBiotech, Melbourne, Australia), visualized, and photographed using the BZ-9000 series Keyence microscope (Keyence GmbH, Neu-Isenburg, Germany), and www.jidonline.org 2384.e1
analyzed using BZ-II Analyzer software (Keyence GmbH). Throughout the manuscript, scale bars in pictures of hematoxylin and eosin staining were taken at ×200 magnification. In pictures of Ly-6G staining, scale bars represent 50 μm and were taken at ×600 magnification.

**Reactive oxygen species release assays upon stimulation with fixed immune complexes**

Fixed immune complexes were generated, as previously described (Sadik et al., 2012). Briefly, immobilized immune complexes were formed using human serum albumin (Merck KGaA, Darmstadt, Germany) and rabbit polyclonal anti-human serum albumin IgG (Merck KGaA). High-binding 96-well ELISA plates (Greiner Bio one, Frickenhausen, Germany) were incubated overnight with 20 μg/ml of human serum albumin in 50 mM carbonate/bicarbonate buffer (pH 9.6). Afterwards, wells were washed with 0.01 M PBS (pH 7.2) and blocked for 1 hour with 10% fetal calf serum in 0.01 M PBS (pH 7.2). Wells were washed again and incubated with anti-human serum albumin Abs diluted in 1/400 in PBS or with PBS only as control for 6 hours. In parallel to the preparation of immune complex-coated plates, neutrophils were isolated from healthy human donor’s blood, using the Polymorphprep protocol (PROGEN Biotechnik GmbH, Heidelberg, Germany). Erythrocytes were lysed by hypotonic PBS (1:5 dilution) for 30 seconds. Then, cells were centrifugated and afterwards resuspended in chemiluminescence medium (RPMI-1640 without phenol red + 1% fetal calf serum + 1 g/ml glucose + 25 mM HEPES). Then neutrophils were adjusted to a density of 10^6 cells/ml in chemiluminescence medium before 0.2 mM luminol was added. A total of 200 μl of this suspension was seeded into each well of the immune complex-coated 96-well plate. Afterwards, cells were incubated at 37 °C in the Infinite M200 PRO ELISA reader (Thermo Fischer Scientific) for 1 hour while the chemiluminescence generated by the luminol reaction, as a surrogate for reactive oxygen species release, was recorded. Afterwards, the area under the curve of the 1-hour time course of chemiluminescence was calculated and used for comparison.

**mRNA analysis of skin samples**

Biopsies of lesional skin were taken when killing the mice and were shock frozen on liquid nitrogen before being stored at −80 °C. Lesional skin biopsies then were embedded in Tissue-Tek Cryomold (VWR, Darmstadt, Germany) before 15 μm sections were cut. These cuts were immediately dissolved in 350 μl of guanidinium-isothiocyanate—containing lysis buffer for isolation of total RNA (RNeasy kit; Qiagen, Hilden, Germany) and were stored at −20 °C. Total RNA was isolated according to the manufacturer’s protocol. To increase the RNA concentration, the final volume of the extracted RNA was reduced to 8 μl (Speedvac concentrator; Eppendorf, Hamburg, Germany). After treatment with DNase I (Merck KGaA, Darmstadt, Germany) for 15 minutes, the RNA was heated 70 °C for 10 minutes and immediately cooled on ice (Kalies et al., 2008). cDNA synthesis was performed with 200 ng of random primer (Promega, Madison, WI), 0.01 M dithiothreitol, 1× reaction buffer, 0.5 mM dNTP (each obtained from Promega), and 100 U reverse transcriptase Superscript II RNase H− (Invitrogen Life Technologies, Carlsbad, CA) in a total volume of 20 μl. Samples were incubated at 42 °C for 50 minutes. No reverse transcriptase enzyme was added to the controls. The cDNA was added to the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and amplified as described above. The TaqMan probes, forward and reverse primers were designed using the computer software CloneManager (Sci-Ed Central, version 7.01). The primer concentrations used for the forward and reverse primers are 900 nM. The TaqMan probes were used in a concentration of 200 nM (Biomers, Ulm).

**Determination of the content of FoxP3+ cells in the skin**

To detect FoxP3+ cells, 6 μm skin paraffin sections were deparaffinized and heat-mediated antigen retrieval was performed in 0.1 M sodium citrate (pH 6) buffer. Subsequently, sections were washed thrice, 5 minutes each time, with 0.01 M PBST (pH 7.2) and treated with 1% (v/v) Triton X-100 in PBS for 30 minutes at room temperature. After washing, sections were blocked for 60 minutes with 5% normal goat serum (NGS; Dako, Glostrup, Denmark) at room temperature. Then, 0.01 mg/ml of primary rat anti-mouse Foxp3 antibody (Thermo Fischer Scientific GmbH, Dreieich, Germany) in 5% (v/v) NGS in PBST or 0.01 mg/ml of total rat IgG (Emret ANALYTICS, Eibelstadt, Germany) were added to sections for overnight incubation at 4 °C. Thereafter, slides were washed with PBST thrice and incubated with 0.0025 mg/ml Alexa-Fluor 594 AffiniPure goat-anti-rat IgG (Jackson ImmunoResearch, Suffolk, United Kingdom) for 1 hour at room temperature. Slides were washed as described before and mounted with DAPI fluoromount G (SouthernBiotech, Birmingham, AL). Images were acquired on Keyence Microscope BZ-9000E series (Keyence GmbH) and analyzed using BZ-II Analyzer (Keyence GmbH). To quantify the degree of FoxP3+ infiltration in the skin, cells were counted in three independent high-power magnification fields (×200) per sample and a mean value was calculated.

For the preparation of total protein skin extracts, skin samples were excised immediately postmortem, snap frozen in liquid nitrogen, and stored at −80 °C until being processed. Skin samples were grinded into a powder using mortar and pestle, weighed on an analytical scale, and vortexed in the presence of T-PER reagent (Thermo Fischer Scientific GmbH) at 1:6 (w/v) in the presence of 1% (v/v) protease inhibitor cocktail set III (Merck, Darmstadt, Germany). Tissue homogenates were centrifuged at 10,000g for 10 minutes at 4 °C to pellet the debris. To minimize unspecific binding because of the presence of murine IgG, supernatants were collected and incubated with A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Dallas, Texas) at a ratio of 6:1 v/v on a shaker at 4 °C. After overnight incubation samples were centrifuged at 1,000g for 5 minutes at 4 °C, and supernatants were collected and stored at −80 °C. Total protein concentration was determined using BCA assay kit (Thermo Fischer Scientific GmbH) according to manufacturer’s instructions. Myeloperoxidase was measured in the supernatants using mouse MPO DuoSet ELISA kit (Bio-Techne,
Wiesbaden-Nordenstadt, Germany) in accordance with manufacturer’s instructions. The obtained concentration of myeloperoxidase was normalized to the total protein content in the measured sample. Then the density of FoxP3\textsuperscript{+} cells was, for each sample, normalized to myeloperoxidase/total protein to normalize for the degree of neutrophil infiltration in the skin.

**Statistical Analysis**

All data are presented as mean ± standard error of the mean or ± standard deviation, as indicated. Statistical differences were determined using GraphPad Prism 7.0 (GraphPad, San Diego, CA) or R analysis of variance. \( P < 0.05 \) was considered statistically significant. Clinical scores were checked for normal distribution using the Shapiro–Wilk test. Afterwards, we performed two-way analysis of variance as implemented in R analysis of variance to access statistical differences among the groups (treatment and control) and across the weeks. The post hoc test was performed using pairwise \( t \)-test and the \( P \)-values were adjusted for multiple testing type I error using Benjamini–Hochberg correction. FACS, ELISA, and quantitative reverse transcriptase in real time results were analyzed by Mann–Whitney test. The \( P \)-adjusted values <0.05 were considered significant.

**SUPPLEMENTARY REFERENCES**


**Supplementary Figure S1.** Fingolimod decreases the abundance of Tregs in relation to neutrophils in lesional skin in the antibody transfer EBA model. Abundance of FoxP3\textsuperscript{+} cells relative to the content of MPO as a marker for neutrophil infiltration in lesional skin of vehicle- and fingolimod-treated mice harvested on day 16 of the antibody transfer model. Results were compared by Mann–Whitney U test \((n = 4 \text{ mice/group})\). EBA, epidermolysis bullosa acquisita; MPO, myeloperoxidase; Treg, regulatory T cell.