



Granzyme B Contributes to Barrier Dysfunction in Oxazolone-Induced Skin Inflammation through E-Cadherin and FLG Cleavage

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Atopic dermatitis (AD) is the most common inflammatory skin condition. Skin barrier dysfunction is of major importance in AD because it facilitates allergen sensitization and systemic allergic responses. Long regarded as a pro-apoptotic protease, emerging studies indicate granzyme B (GzmB) to have extracellular roles involving the proteolytic cleavage of extracellular matrix, cell adhesion proteins, and basement membrane proteins. Minimally expressed in normal skin, GzmB is elevated in AD and is positively correlated with disease severity and pruritus. We hypothesized that GzmB contributes to AD through extracellular protein cleavage. A causative role for GzmB was assessed in an oxazolone-induced murine model of dermatitis, comparing *GzmB*^{-/-} mice with wild-type mice, showing significant reductions in inflammation, epidermal thickness, and lesion formation in *GzmB*^{-/-} mice. Topical administration of a small-molecule GzmB inhibitor reduced disease severity compared with vehicle-treated controls. Mechanistically, GzmB impaired epithelial barrier function through E-cadherin and FLG cleavage. GzmB proteolytic activity contributes to impaired epidermal barrier function and represents a valid therapeutic target for AD.

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INTRODUCTION

Granzyme B (GzmB) is a serine protease traditionally known for its role in lymphocyte-mediated apoptosis. GzmB is secreted toward the target cell along with the pore-forming protein perforin, which facilitates entry and initiation of the apoptotic cascade (Masson and Tschopp, 1987). However, during effector-target cell engagement, approximately one-third of GzmB escapes from the immunological synapse and into the extracellular milieu (Isaaz et al., 1995) where it retains its activity. Moreover, other types of immune cells as well as nonimmune cells are now known to express and secrete GzmB, including mast cells (Pardo et al., 2007) and

keratinocytes (KCs) (Hernandez-Pigeon et al., 2006), with many unable to express perforin and/or form immunological synapses with target cells. Taken together, GzmB expression by nontraditional cells and the presence of proteolytically active GzmB in the extracellular milieu suggests additional noncytotoxic roles.

Although the expression is negligible in healthy tissue, GzmB is dramatically upregulated in a range of cutaneous conditions, including autoimmune and bullous disorders (Bodemer et al., 2000; Russo et al., 2018), diabetic ulcers (Hsu et al., 2014), and photo-damaged skin (Parkinson et al., 2015). However, the intracellular and extracellular contribution of GzmB appears to vary according to the disease type and is likely dependent on the context of where and when GzmB is produced (reviewed in Boivin et al., [2009]). As of now, no endogenous inhibitors of extracellular GzmB have been identified in human biofluids, and GzmB retains enzymatic activity in human plasma (Kurschus et al., 2004); thus, GzmB appears to act unimpeded in damaged tissues. Extracellular GzmB may contribute to the pathogenicity observed in many inflammatory skin diseases through the direct cleavage of the extracellular matrix, cell junction, and/or basement membrane proteins (reviewed in Turner et al., [2019]), many of which play an important role in skin function and integrity.

Atopic dermatitis (AD) is the most common inflammatory skin condition, with skin barrier dysfunction being of major importance because it facilitates allergen sensitization and systemic allergic responses (Kim and Leung, 2018). In the plasma from patients with AD, GzmB is elevated compared with plasma from healthy controls, with plasma GzmB concentration positively correlated with markers of AD severity

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Abbreviations: AD, atopic dermatitis; AUC, area under the curve; GzmB, granzyme B; DSG, desmoglein; IHC, immunohistochemistry; KC, keratinocyte; OXA, oxazolone; OXA-dermatitis, OXA-induced dermatitis; SC, stratum corneum; sE-Cadherin, soluble E-cadherin; TBO, toluidine blue; TEWL, transepidermal water loss; WT, wild type

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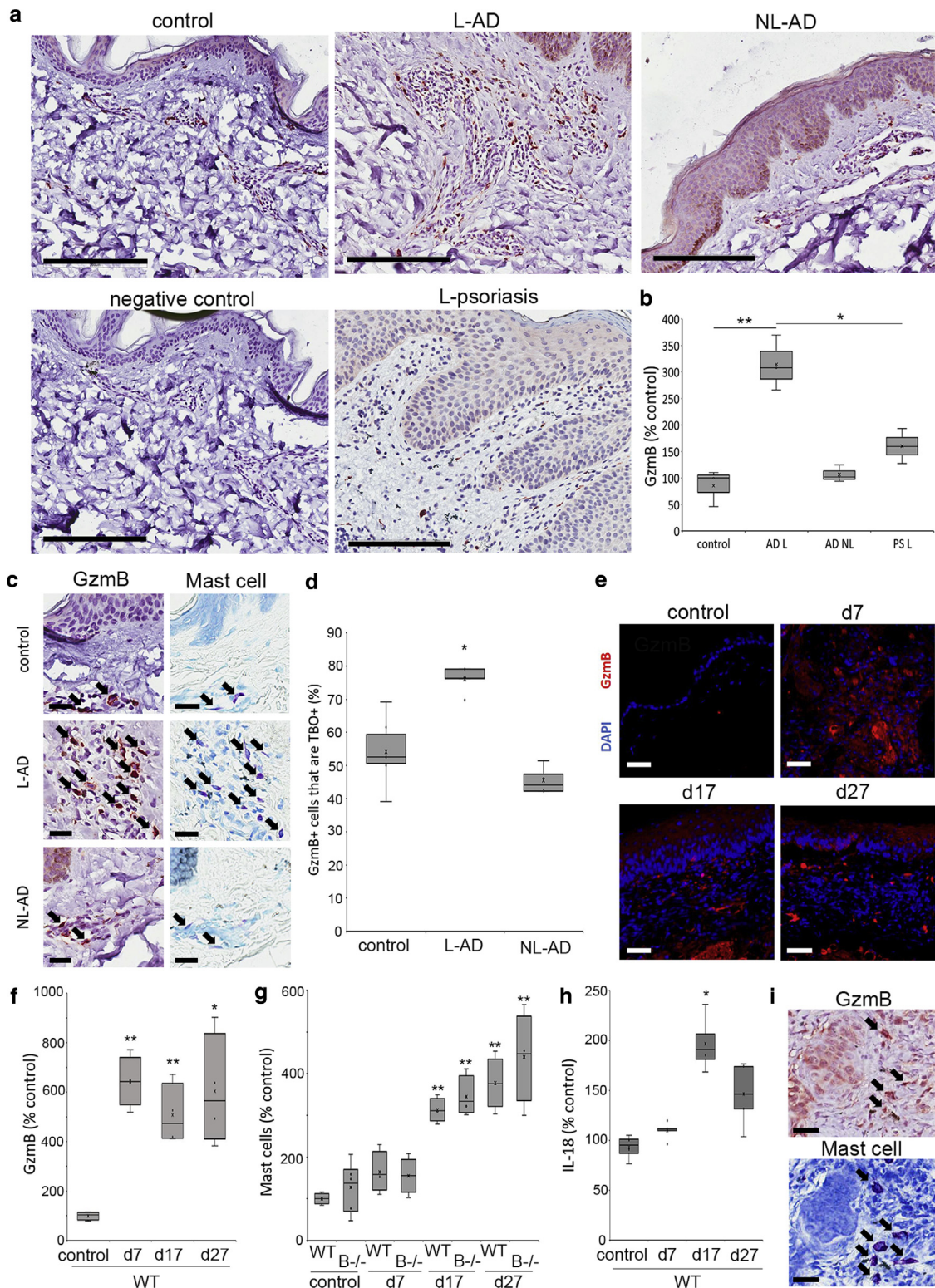


Figure 1. GzmB is elevated in AD in human and colocalizes to mast cells. (a) GzmB expression in AD in human. (b) GzmB⁺ cell quantification. (c) Serial GzmB and TBO stain (mast cell marker) in AD tissue in human. Arrows indicate cells for both TBO⁺ and GzmB⁺. (d) Percentage of TBO⁺ cells that were GzmB⁺. (e) GzmB immunofluorescence and (f) quantification in the skin of mouse with OXA-dermatitis. (g) TBO⁺ cell quantification in OXA-dermatitis ears. (h) IL-18 quantification in ears of WT mouse with OXA-dermatitis. (i) GzmB⁺ and TBO⁺ cell detection in serial sections of OXA-dermatitis ears on day 17. Arrows indicate GzmB⁺ or TBO⁺ cells. Data were analyzed by two-way ANOVA with Bonferroni post-hoc test. **P* < 0.05, ***P* < 0.005, *n* = 4 per group. Bars are (a, e) 200 μ m, (c) 5 μ m, and (i) 30 μ m. Representative images are shown. AD, atopic dermatitis; d, day; GzmB, granzyme B; L-AD, lesional AD; L-psoriasis, lesional psoriasis; NL-AD, nonlesional AD; OXA, oxazolone; OXA-dermatitis, OXA-induced dermatitis; PS L, lesional psoriasis; TBO, toluidine blue; WT, wild type.

(Kamata et al., 2016). A positive correlation is established between plasma GzmB concentration and Severity Scoring AD, a clinical index tool used to assess the extent and severity of AD. Plasma levels of the itch-related peptide, gastrin-releasing peptide, typically elevated in patients with AD also positively correlate with plasma GzmB concentration. Furthermore, GzmB expression is elevated in AD lesional skin compared with healthy and nonlesional skin (Zhang et al., 2015). Saliently, no correlations were evident among GzmB levels, Visual Analogue Scale score, and PASI score in patients with psoriasis, suggesting GzmB to specifically play an important role in AD (Kamata et al., 2016).

Despite the clear link between GzmB and AD severity, a functional pathologic role for GzmB remains elusive. In this study, mast cells were shown to be upregulated and express GzmB in lesional tissue from human subjects and in mice with hapten-mediated dermatitis, contributing to increased disease severity through the cleavage of proteins instrumental in maintaining an intact barrier function.

RESULTS

GzmB is elevated in AD lesions in humans and primarily expressed by mast cells

GzmB expression was assessed in AD samples from humans, comparing lesional and nonlesional skin derived from the same patients with psoriatic skin and healthy skin controls (Supplementary Table S1). Elevated cell-specific GzmB immune positivity was observed throughout the dermis of lesional AD compared with that of nonlesional AD and healthy skin ($P = 0.0096$, $n = 3$) predominantly in regions of inflammatory cell infiltrate (Figure 1a and b). Unlike AD, GzmB immune positivity was not elevated in lesional psoriatic skin compared with healthy skin, in agreement with a previous study (Yawalkar et al., 2001a). Extracellular GzmB could not be assessed histologically owing to a lack of assay sensitivity.

Mast cells, which secrete GzmB (Pardo et al., 2007) and are elevated in AD (Kawakami et al., 2009), were elevated in lesional AD compared with nonlesional and healthy tissue (Figure 1c). Sequential staining of lesional AD, nonlesional AD, and healthy skin demonstrated that all toluidine blue⁺ (TBO⁺ [mast]) cells also express GzmB. Interestingly, in lesional AD, significantly more GzmB⁺ cells were TBO⁺ (75%) than observed in both nonlesional and healthy skin (45–50%, $P = 0.0196$; Figure 1d and Supplementary Figure S1).

GzmB is elevated in mice with oxazolone-induced dermatitis

Hapten-mediated dermatitis was induced in the ears of *GzmB*^{−/−} and wild-type (WT) mice using repeated applications of 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone [OXA]) (Supplementary Figure S2a) as described in Park et al., (2011). Continued OXA challenges are reflective of AD that is human-like, and although haptenized proteins are not typically formed in AD in humans, it does induce chronic inflammation and crucially for the current investigation, a conversion from a T helper type 1 to T helper type 2 response (confirmed in WT mice for this study; Supplementary Figure S3), increased mast cell recruitment,

elevated serine protease activity, and barrier disruption (Jin et al., 2009; Man et al., 2008).

OXA-induced dermatitis (OXA-dermatitis) lesions in mice displayed 6-fold increased GzmB detection compared with untreated mice predominantly localized to the regions of inflammatory cell infiltrate, similar to AD in humans ($P < 0.005$ on day 7 and day 17 and $P < 0.05$ on day 27; Figure 1e and f). Mast cells were elevated in OXA-dermatitis lesions compared with controls ($P < 0.005$ on day 17 and day 27; Figure 1g) and corresponded to increased detection of the proinflammatory cytokine IL-18 (Figure 1h), which is reported to stimulate mast cells to secrete chymase (and likely other secreted proteases, including GzmB) (Tsutsui et al., 2004), leading to accelerated inflammation in AD. Again, similar to lesional AD in humans (Kawakami et al., 2009), mast cells were the primary source of GzmB (Figure 1i). No difference in the number of mast cells between *GzmB*^{−/−} and WT mice were observed at each time point, suggesting that GzmB has no direct effect on mast cell recruitment. The mouse with OXA-dermatitis provides a useful model to study AD in the context of mast cell recruitment and GzmB upregulation.

Reduced severity of OXA-dermatitis in *GzmB*^{−/−} mice

Ear thickness, an indicator of inflammation, was reduced in *GzmB*^{−/−} mice with OXA-dermatitis compared with WT mice (area under the curve [AUC], $P < 0.0001$; Figure 2a). On day 7, lesional ear coverage was reduced in *GzmB*^{−/−} mice compared with WT mice ($P < 0.005$; Figure 2b and c). Epidermal thickness was also reduced in *GzmB*^{−/−} mice compared with WT mice on both days 7 ($P < 0.005$) and 17 ($P < 0.05$; Figure 2b and d). A grading scale was implemented to assess dermatitis severity (Supplementary Table S2), which was adapted from the Severity Scoring AD index and Three-Item Severity score (Kunz et al., 1997; Oranje, 2011). *GzmB*^{−/−} mice had reduced combined severity (measuring combined scaling and/or dryness, erythema, erosion, and alopecia) compared with WT mice (AUC, $P < 0.0001$; Figure 2e). Scaling (AUC, $P < 0.0001$), erythema (AUC, $P = 0.037$), erosion (AUC, $P = 0.0026$), and alopecia (AUC, $P < 0.0001$) were all improved in *GzmB*^{−/−} mice compared with WT controls (Figure 2f–j). The largest difference between *GzmB*^{−/−} mice ears and WT mice ears occurred from approximately day 15, suggesting a temporal effect of GzmB in this animal model and possibly in response to the T helper type 1 to T helper type 2 transition.

GzmB impairs skin barrier function in mice with OXA-dermatitis

Reduced barrier function is a major contributor to AD pathology (reviewed by Cork et al., [2009]). The effect of GzmB on skin barrier function was therefore assessed in the ears of mice with OXA-dermatitis through the measure of trans-epidermal water loss (TEWL). Exposure to OXA led to an increased TEWL compared with nonchallenged ear controls ($P < 0.005$, Figure 3). *GzmB*^{−/−} OXA mice displayed a pronounced reduction in TEWL compared with WT OXA mice (AUC, $P = 0.0012$), with the greatest effect observed between day 4 and day 16 ($P < 0.05$).

HaCaT KCs, which are well-characterized as establishing a strong barrier as measured by Electric Cell-Substrate

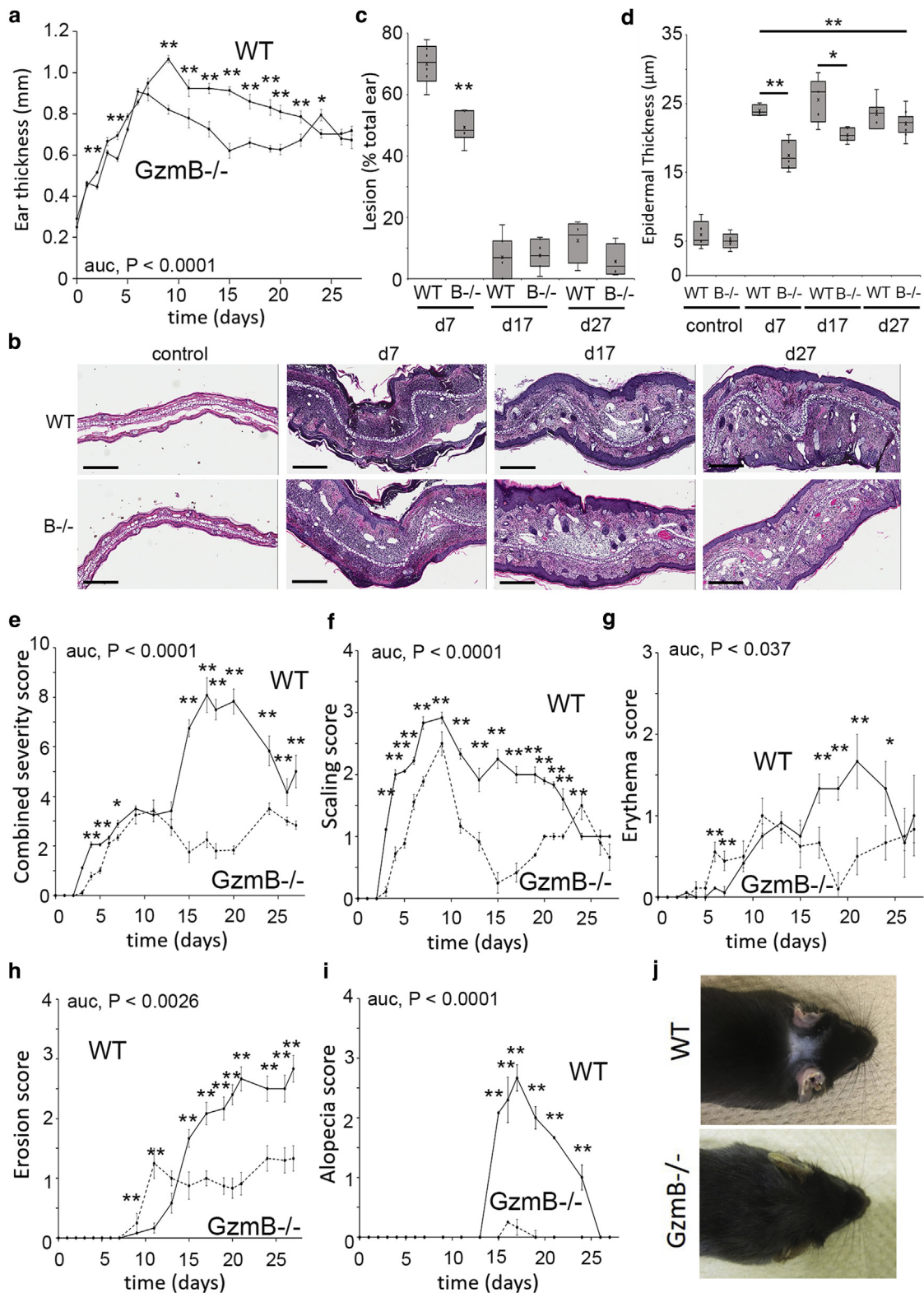


Figure 2. *GzmB*^{-/-} mice with OXA-dermatitis show reduced pathology. (a) Ear thickness in mice with OXA-dermatitis (*n* $\geq$ 12). Representative H&E-stained (b) ear sections, with (c) lesional coverage and (d) epidermal thickness quantified. Bar = 300 μ m. Clinical severity showing (e) combined scores in addition to (f) scaling, (g) erythema, (h) erosion, and (i) alopecia. (j) Photos of mice on day 17 after OXA challenge. Data in a and e–i were analyzed by Student's *t*-test (two-sided, nonpaired) and presented as mean $\pm$ SEM, with AUC also calculated. Data in c and d were analyzed by two-way ANOVA with Bonferroni post-hoc test and presented as a box and whisker plot. **P* < 0.05, ***P* < 0.005, *n* = 4 per group. AUC, area under the curve; d, day; GzmB, granzyme B; OXA, oxazolone; OXA-dermatitis, OXA-induced dermatitis; WT, wild type.

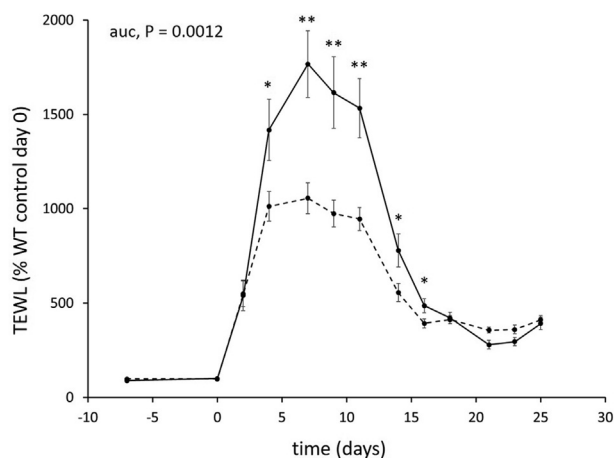


Figure 3. *Gzmb*^{-/-} mice with OXA-dermatitis show improved barrier function. TEWL was determined on the surface of the ears from WT with OXA-dermatitis (solid line) and *Gzmb*^{-/-} (dashed line) mice using a VapoMeter. Data were analyzed by two-way ANOVA with Bonferroni post-hoc test and presented in percentage WT on day 0 as mean $\pm$ SEM, with AUC also calculated. * $P < 0.05$, ** $P < 0.005$, $n > 10$ per group. AUC, area under the curve; Gzmb, granzyme B; OXA, oxazolone; OXA-dermatitis, OXA-induced-exposed dermatitis; TEWL, transepidermal water loss; WT, wild type.

Impedance Sensing (Évora et al., 2017; Huang et al., 2016; Le Gall et al., 2016; Triplett et al., 2019) and other transepidermal resistance assays (Leguina-Ruzzi and Valderas, 2016; Ohnemus et al., 2008; Ramadan and Ting, 2016), were exposed to Gzmb and the barrier subsequently measured. Using Electric Cell-Substrate Impedance Sensing, confluent HaCaT monolayers displayed a dose-dependent reduction in barrier function ($P < 0.005$, 150–200 nM Gzmb) compared with vehicle-treated controls (Supplementary Figure S4a–c). The Gzmb inhibitor compound 20 ameliorated the negative impact of Gzmb on barrier function. An increase in the paracellular flow of 70 kDa rhodamine B-labeled dextran beads in the presence of Gzmb confirmed Gzmb as impairing barrier function in KCs ($P < 0.005$; Supplementary Figure S4d). Gzmb had no effect on cell viability (Supplementary Figure S4e).

Gzmb-mediated cleavage of E-cadherin in mice with OXA-dermatitis

The epidermal junction protein E-cadherin is reduced in AD-affected skin (Salimi et al., 2013; Trautmann et al., 2001), with Gzmb-mediated cleavage potentially contributing to this reduction. In AD lesions of humans, we confirmed reduced E-cadherin intensity (Figure 4a). In vitro, Gzmb triggered a dose-dependent reduction in E-cadherin and the generation of lower molecular weight fragments (Figure 4b). Preincubation of Gzmb with VTI-1002, a potent and specific small molecule Gzmb inhibitor, partially inhibited cleavage. Cultured HaCaTs displayed strong E-cadherin fluorescence in the junctional regions surrounding the cells (Figure 4c) as demonstrated previously in HaCaTs and primary KCs (Maretzky et al., 2005; Ohnemus et al., 2008; Rhys et al., 2018). When exposed to 100 nM Gzmb for 8 hours, a more granular and/or diffuse pattern was observed, with an overall reduction in fluorescence intensity. In addition, there

was an increase in soluble E-cadherin (sE-cadherin) fragments in the culture supernatants (Figure 4d and e). Fragments were released into the culture medium in a dose-dependent manner ($P < 0.005$), with preincubation of VTI-1002 with Gzmb partially inhibiting this response. Both *Gzmb*^{-/-} mice with OXA-dermatitis and WT mice exhibited reductions in E-cadherin in the epidermis compared with the control skin (Figure 4f and g). Saliently, E-cadherin was significantly reduced in WT mice compared with *Gzmb*^{-/-} mice ($P < 0.05$ on day 7), suggesting Gzmb-mediated E-cadherin cleavage. To confirm this, E-cadherin immunohistochemistry (IHC) was performed in ex vivo human skin, with the tissue incubated with Gzmb displaying reduced staining intensity (Figure 4h). Preincubation of Gzmb with VTI-1002 before exposure to the ex vivo skin ameliorated the effect of Gzmb on E-cadherin detection.

Gzmb-mediated cleavage of FLG in mice with OXA-dermatitis

The filament aggregation protein FLG is critical in maintaining epidermal homeostasis, and its deficiency plays an important role in the pathogenesis of AD, in part, through attenuation of barrier functionality (Cabanillas and Novak, 2016). In AD lesions of humans, FLG was reduced in the stratum corneum (SC) compared with the SC in healthy skin (Figure 5a). Incubation with Gzmb led to efficient cleavage of the recombinant human FLG peptide into low molecular weight fragments (Figure 5b). Preincubation of Gzmb with the inhibitor VTI-1002 completely ameliorated this effect. FLG staining intensity was significantly reduced in the SC of WT mice with OXA-dermatitis at each time point compared with that of the control skin ($P < 0.005$; Figure 5c and d), whereas *Gzmb*^{-/-} mice exhibited significantly less FLG degradation on days 7 and 17 ($P < 0.05$). To confirm this, FLG was measured in ear extracts by western blot, showing less FLG monomer in WT mice affected with dermatitis compared with that seen in *Gzmb*^{-/-} mice and reaching statistical significance on day 17 ($P < 0.05$; Figure 5e and f). In the ex vivo human skin exposed to Gzmb, FLG detection was reduced, with this, effect decreased when Gzmb was preincubated with VTI-1002 (Figure 5g).

Gzmb-mediated cleavage of desmoglein-1 in mice with OXA-dermatitis

Although E-cadherin and FLG are important in maintaining epidermal barrier function in the skin and the focus of much research in AD, other intercellular proteins may also be cleaved by Gzmb and contribute to AD pathogenesis. In vitro cleavage assays showed ZO-1, JAM-A, desmoglein (DSG)-3 (Supplementary Figure S5), and DSG-1 (Supplementary Figure S6) to each be cleaved by Gzmb, with this corresponding to reduced and more diffuse immune detection in cultured KCs. DSG-1 staining intensity was significantly reduced in the epidermis of WT mice with OXA-dermatitis at each time point compared with the epidermis of the control skin ($P < 0.005$; Supplementary Figure S6d and e), whereas *Gzmb*^{-/-} mice exhibited significantly less DSG-1 degradation on days 17 and 27 ($P < 0.05$). There was no difference in the detection of ZO-1, JAM-A, and DSG-3 between WT and *Gzmb*^{-/-} mice with OXA-dermatitis (data not shown).

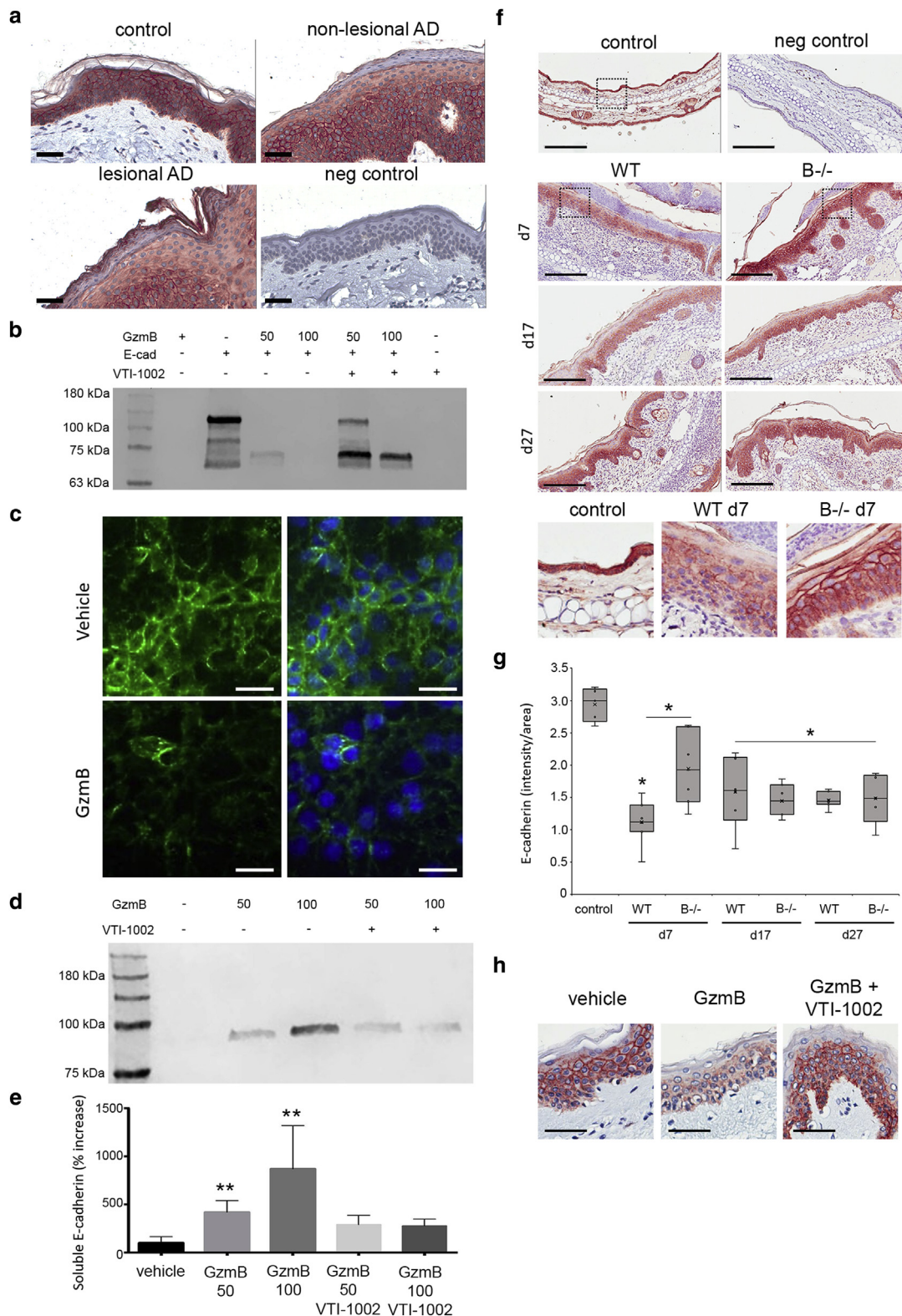


Figure 4. Reduced GzmB-mediated E-cadherin cleavage in *GzmB*^{-/-} mice with OXA-Dermatitis. (a) E-cadherin in the epidermis of AD in human. (b) Biochemical cleavage of purified recombinant human E-cadherin with GzmB ± VTI-1002 and visualized by western blot. (c) E-cadherin immunofluorescence (green) in HaCaT at confluence ± 100 nM GzmB or vehicle for 8 hours. Blue = DAPI. (d) Detection and (e) semiquantification of GzmB-mediated sE-cadherin cleavage fragments in HaCaT supernatants. (f) E-cadherin detection and (g) quantification in the epidermis of OXA-dermatitis ears. (h) E-cadherin detection in ex vivo human skin exposed to GzmB ± VTI-1002. Data were analyzed by two-way ANOVA with Bonferroni post-hoc test and presented as (e) mean ± SD or (g) box and whisker plot. **P* < 0.05, ***P* < 0.005, *n* = 4 per group. Bars are (a) 50 μm, (c) 30 μm, (f) 200 μm, and (h) 60 μm, with representative images shown. AD, atopic dermatitis; d, day; E-cad, E-cadherin; GzmB, granzyme B; neg, negative; OXA, oxazolone; OXA-dermatitis, OXA-induced dermatitis; sE-cadherin, soluble E-cadherin; WT, wild type.

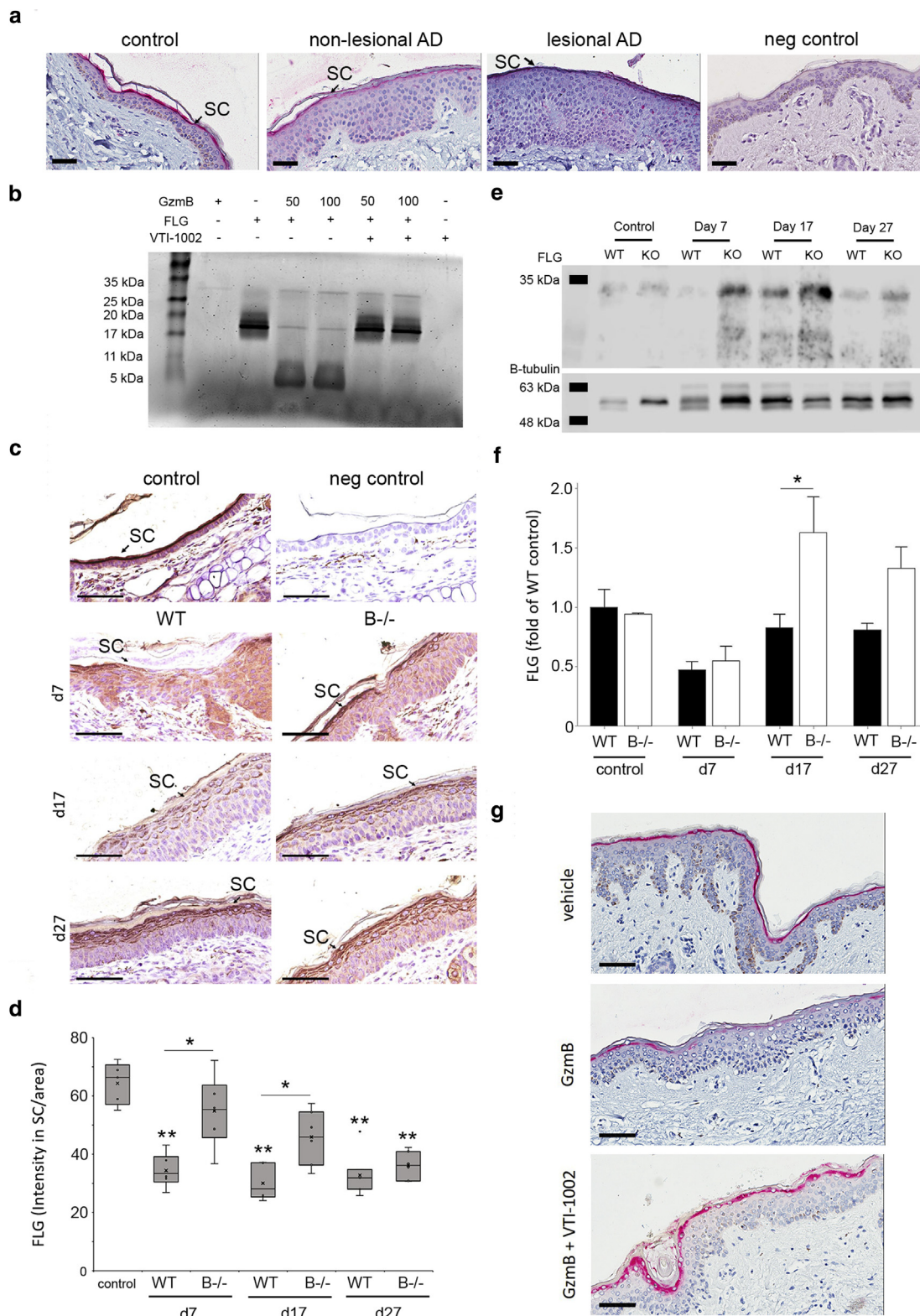


Figure 5. Reduced GzmB-mediated FLG cleavage in *GzmB*^{-/-} mice with OXA-dermatitis. (a) FLG in the SC of AD tissue in human. (b) Biochemical cleavage of recombinant partial sequence FLG (3838–4061 aa) with GzmB ± VTI-1002 and visualized by Coomassie stain. (c) FLG detection and (d) quantification in the SC of OXA-dermatitis ears. FLG expression analyzed by (e) western blot and (f) semiquantified in OXA-dermatitis ear extracts (n = 4). β -Tubulin was included as a loading control, with a representative sample included in this blot. (g) FLG detection in ex vivo human skin exposed to GzmB ± VTI-1002. Data were analyzed by two-way ANOVA with Bonferroni post-hoc test and presented in d as box and whisker plot and f as mean $\pm$ SD. * P < 0.05, ** P < 0.005, n = 4 per group. Bars are (a) 50 μ m, (c) 200 μ m, and (g) 70 μ m, with representative images shown. AD, atopic dermatitis; d, day; GzmB, granzyme B; KO, knockout; neg, negative; OXA, oxazolone; OXA-dermatitis, OXA-induced dermatitis; SC, stratum corneum; WT, wild type.

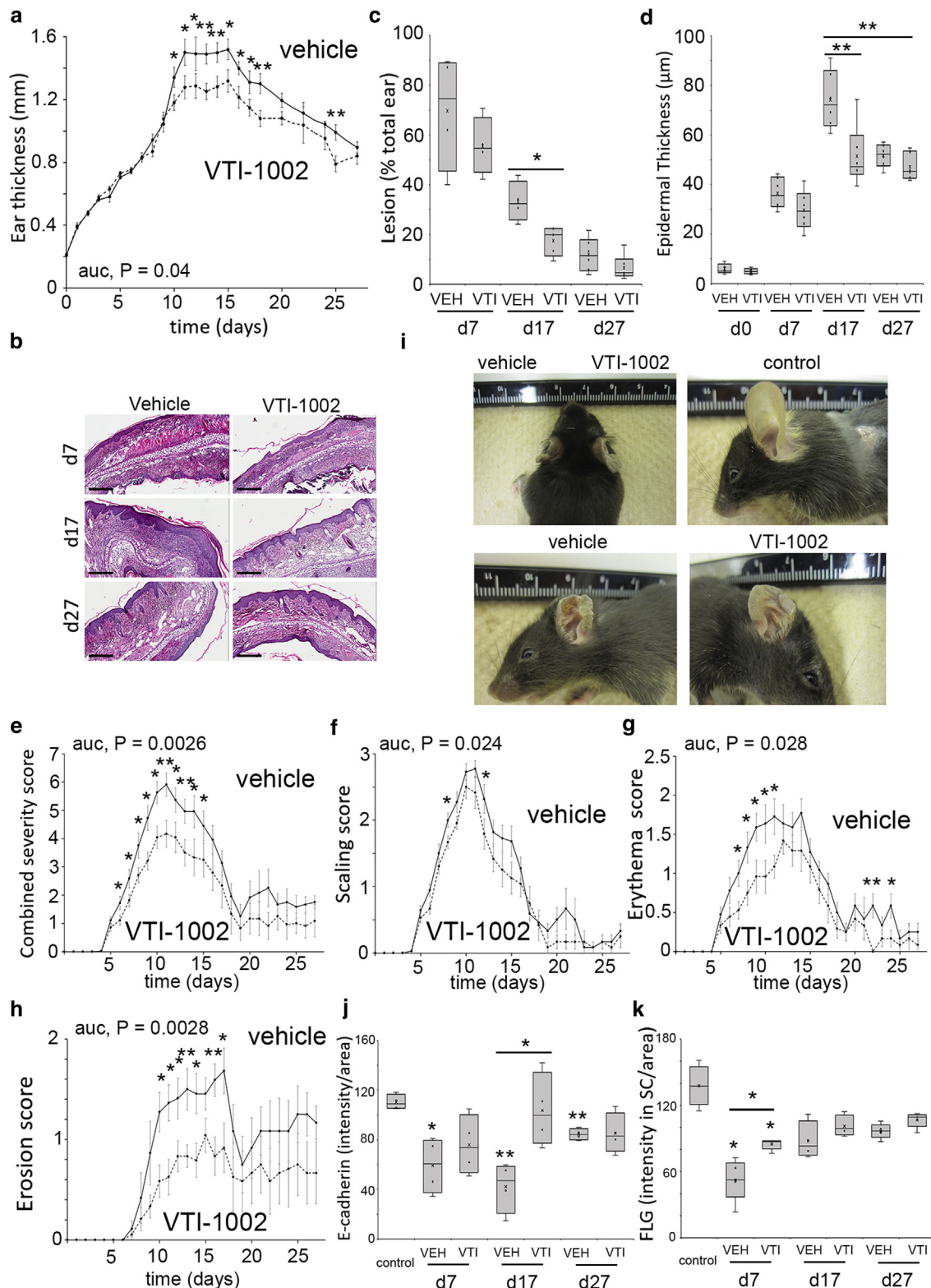


Figure 6. Topical GzmB inhibitor improves OXA-dermatitis in WT mice by reducing E-cadherin and FLG cleavage. (a) Ear thickness in VTI-1002-treated WT mice with OXA-dermatitis. One ear per mouse treated with VTI-1002, with vehicle administered to the other. Representative H&E-stained (b) ear sections with (c) lesional coverage and (d) epidermal thickness quantified. Bar = 300 μm . Combined (e) severity scores, (f) scaling, (g) erythema, and (h) erosion. (i) Mice photos on day 27 after OXA challenge. (j) FLG and (k) E-cadherin quantification in OXA-dermatitis ears (representative images are presented in [Supplementary Figure S8](#)). Data were analyzed by two-way ANOVA with Bonferroni post-hoc test and presented in a and e–h as mean $\pm$ SEM and c, d, j, k as box and whisker plot. * $P < 0.05$, ** $P < 0.005$, $n > 6$. AUC, area under the curve; d, day; GzmB, Granzyme B; OXA, oxazolone; OXA-dermatitis, OXA-induced dermatitis; SC, stratum corneum; VEH, vehicle; VTI, VTI-1002; WT, wild type.

GzmB-mediated cleavage of decorin in mice with OXA-dermatitis

GzmB-mediated cleavage of decorin occurs in various wound models and contributes to impaired extracellular matrix remodeling (reviewed in Turner et al., [2019]), thus may provide an additional contribution to AD pathogenesis. Samples of AD from human (Supplementary Figure S7a) and WT mice with OXA-dermatitis ($P < 0.005$, Supplementary Figure S7b and c) each exhibited reduced decorin detection in the dermis compared with control skin; this decrease was not as pronounced in *GzmB*^{-/-} mice with OXA-dermatitis (Supplementary Figure S7b and c). This corresponded to improved collagen organization in the *GzmB*^{-/-} mice (Supplementary Figure S7d and e), suggesting that GzmB contributes to impaired skin structural integrity and overall strength in AD.

GzmB-mediated cleavage of fibronectin is reported in a mouse model of contact dermatitis and contributes to elevated vascular permeability (Hendel et al., 2014) while also inducing matrix metalloproteinase-1 release from fibroblasts and thereby facilitating increased collagen turnover (Parkinson et al., 2015). This is exacerbated in response to GzmB-mediated decorin cleavage as collagen becomes more susceptible to matrix metalloproteinase-1-mediated degradation (Gubbiotti et al., 2016; Parkinson et al., 2015). In the skin of mice with OXA-dermatitis, fibronectin was reduced at all time points; however, there was no dramatic difference between *GzmB*^{-/-} and WT mice with OXA-dermatitis (Supplementary Figure S8). In addition, there was no apparent difference in the expression of matrix metalloproteinase-1 between *GzmB*^{-/-} and WT mice with OXA-dermatitis (data not shown).

VTI-1002 reduces severity in WT mice with OXA-dermatitis

WT mice with OXA-dermatitis were administered a topical formulation of VTI-1002 to evaluate its capacity to improve lesional severity (Supplementary Figure S2b). Ear thickness (AUC, $P = 0.04$; Figure 6a), lesional coverage ($P < 0.05$ on day 17; Figure 6b and c), and epidermal thickness ($P < 0.005$ on day 17; Figure 6b and d) were reduced in VTI-1002-treated ears compared with vehicle-treated ears. Overall ear appearance was also improved (AUC, $P = 0.0026$; Figure 6e and i), with erythema (AUC, $P = 0.028$) and erosions (AUC, $P = 0.0028$) showing the greatest improvement in response to VTI-1002 (Figure 6g and h). Only a small reduction in dryness and/or scaling (AUC, $P = 0.024$) was observed (Figure 6f) likely due to the moisturizing effect of the vehicle. Alopecia was not examined because VTI-1002 was only applied to the ears. VTI-1002 treatment decreased the dermatitis-mediated reduction of E-cadherin within the epidermis ($P < 0.05$ on day 17; Figure 6j and Supplementary Figure S9) and FLG in the SC ($P < 0.05$ on day 7; Figure 6k and Supplementary Figure S9) compared with vehicle treatment.

DISCUSSION

Despite being elevated and correlated with pruritus and disease severity (Kamata et al., 2016; Yawalkar et al., 2001b), the exact role of GzmB in the pathogenesis of AD remains to be elucidated. In this study, mast cells were identified as a primary cell source responsible for elevated GzmB in AD in humans and mice with OXA-dermatitis. Previous studies in

AD (Yawalkar et al., 2001b) and contact dermatitis (Yawalkar et al., 2001a), performed when GzmB was believed to operate exclusively in perforin-dependent cytotoxicity, identified GzmB to be expressed by CD4⁺ and CD8⁺ cells. However, these studies were performed before our current understanding of the extracellular roles for GzmB; therefore, GzmB was not directly measured and/or assessed in other cell types, including those important in AD. In contact dermatitis (Yawalkar et al., 2001a) and other inflammatory skin diseases (Schmid et al., 2002), more GzmB⁺ cells were present than perforin⁺ cells, supporting an extracellular role for GzmB. In fact, Yawalkar et al. (2001b) was unable to identify a major cell source of GzmB⁺ cells, speculating them to be macrophages.

Mast cells are upregulated and have an important pathologic role in dermatitis-affected skin, including AD (Kawakami et al., 2009) and chronic allergic contact dermatitis (Gimenez-Rivera et al., 2016). Importantly, mast cells are known to express and secrete GzmB (Pardo et al., 2007). Skin-derived mast cells secrete GzmB, possibly in response to IL-18 exposure, and function independently of perforin (Pardo et al., 2007). Together, extracellular GzmB and the established cytotoxic role for GzmB, may both contribute to the pathogenesis of AD. Further studies are required to elucidate the contributions of each in AD as well as other inflammatory skin diseases. In addition, more study is required to understand the specific contribution of mast cell-derived GzmB to disease pathogenesis, especially as other immune and nonimmune cell types are known to express GzmB (reviewed in Turner et al., [2019]).

The mouse model of OXA-dermatitis is associated with localized spongiotic dermatitis analogous to a chronic contact allergic reaction but also reflects multiple pathologic features characteristic of AD seen in humans (Jin et al., 2009; Man et al., 2008; Martin, 2013; Tanaka et al., 2012; Zachariassen et al., 2017), including a number of features critical to the study of GzmB upregulation and barrier dysfunction. Unlike a number of alternative murine models of AD, mice with OXA-dermatitis display increased mast cell recruitment, increased barrier permeability, abnormal barrier protein expression, and SC defects (Man et al., 2008). In this study, WT mice with OXA-dermatitis displayed increased GzmB expression in lesional skin, localizing similarly to lesional AD in human. This comparable tissue expression and localization in both mice and human subjects supported the hapten-mediated dermatitis model as a useful tool to study a potential causative role for GzmB in AD barrier dysfunction in humans.

GzmB^{-/-} mice with OXA-dermatitis exhibited reduced overall inflammation, ear thickness, lesion size, and epidermal thickness compared with WT mice with OXA-dermatitis. In addition, there was less scaling, fewer erosions, reduced erythema, and an almost complete absence of alopecia, likely in response to the overall reduction in inflammation. Treatment of WT mice with OXA-dermatitis with a topical small-molecule GzmB inhibitor similarly improved disease severity compared with the vehicle-treated controls. As such, GzmB appears to contribute to a number of key elements that typify AD pathology.

Serine proteases have an important role in epidermal barrier homeostasis (Hachem et al., 2006; Man et al., 2008),

with pharmacological inhibition shown to accelerate barrier recovery after disruption (Hachem et al., 2006). As of now, the role of GzmB-mediated proteolysis in the epidermal barrier function remains unknown. The ability of extracellular GzmB to disrupt barrier function was therefore investigated, showing increased TEWL in the skin of mice OXA-dermatitis in the presence of GzmB.

In this study, E-cadherin, a transmembrane glycoprotein that connects KCs together at adherens junctions, was found to be cleaved by GzmB, whereas in KC culture, E-cadherin cleavage directly correlated to a dose-dependent impairment of barrier function. The epidermis of ears of *GzmB*^{-/-} mice with OXA-dermatitis and VTI-1002-treated mice ears displayed stronger E-cadherin detection than the epidermis of the WT controls and together suggested GzmB as contributing to E-cadherin cleavage and AD pathogenesis by impairing barrier function. GzmB-mediated cleavage of E-cadherin also generated sE-cadherin fragments. sE-cadherin fragments are elevated in dermatitis (Grabowska and Day, 2012) and reportedly affect cell–cell adhesion, cell migration (van Roy and Berx, 2008), and disruption of preformed adherens junctions (Symowicz et al., 2007). sE-cadherin also disrupts cell–cell junctions and therefore decreases barrier function (Hu et al., 2016). GzmB may therefore further contribute to AD pathogenesis through sE-cadherin-mediated barrier dysfunction.

Mutations in the human *FLG* gene are a major cause of AD (Sandilands et al., 2009). However, a significant number of patients with AD lacks *FLG* mutations; thus, other factors are expected to mediate reduced FLG levels, with these being critically important to AD pathogenesis (O'Regan et al., 2008). In WT mice with OXA-dermatitis, reduced FLG within the SC was observed compared with that in the control skin; however, both the *GzmB*^{-/-} and VTI-1002-treated mice had less FLG loss, suggesting that GzmB cleaved FLG within the affected tissue. Whether mast cell-derived GzmB is responsible for the cleavage of FLG in OXA-dermatitis remains to be elucidated and is the focus of further study. GzmB is reported to be expressed by KCs (Hernandez-Pigeon et al., 2006); thus, this cell source of GzmB may be responsible for FLG cleavage. Therapies focused on the prevention of FLG loss and/or restoration of FLG are predicted to maintain and/or reestablish skin barrier and provide clinical benefit to patients with AD (Cabanillas and Novak, 2016). By cleaving FLG, GzmB appears to have an important role in the pathogenicity of AD and implies that inhibiting GzmB-mediated FLG cleavage could act to maintain barrier function.

Topically applied VTI-1002 has previously been shown to improve burn wound healing (Shen et al., 2018). Topical VTI-1002 penetrated the SC, was retained up to 24 hours, and was well-tolerated in vivo with no adverse events observed when applied daily over a 30-day period. In this study, VTI-1002 exhibited strong efficacy, displaying improved overall ear appearance, reduced ear thickness, improved lesions, reduced epidermal thickness, improved erythema, and fewer erosions. The small-molecule inhibitor appeared to work by preventing and/or reducing GzmB-mediated cleavage of FLG and E-cadherin. Interestingly, dermatitis in WT mice (no treatment) was more severe than that observed in vehicle-

treated WT mice. This may be explained by the skin moisturizing effect of the topically applied vehicle, which was expected to minimize the degree of barrier impairment. Preventing impaired barrier permeability has been identified as a major challenge in therapy development (Cabanillas and Novak, 2016). Given that VTI-1002 inhibits human GzmB 40-fold stronger than mouse GzmB (Shen et al., 2018), this drug would be predicted to have even greater efficacy in human subjects.

To our knowledge, the present study links elevated GzmB in AD in human to proof-of-concept and mechanism of action in mice. GzmB-mediated cleavage increases permeability through both major skin barriers that are typically disrupted in AD, namely cell–cell adhesion proteins, including E-cadherin in the epidermis, and FLG within the SC. Therefore, GzmB represents a valid therapeutic target for the treatment of AD.

MATERIALS AND METHODS

Biochemical cleavage assay

E-cadherin, FLG (partial sequence), DSG-1, DSG-3 (partial sequence), ZO-1 (partial sequence), and JAM-A were separately incubated (20 µg/ml) with 50–100 nM GzmB in 50 mM tris(hydroxymethyl)aminomethane-hydrochloric acid, pH 7.4 buffer for 2 hours at 37 °C (Supplementary Table S3). GzmB was preincubated with 50 µM VTI-1002 (viDA Therapeutics, Vancouver, BC), E-cadherin, FLG, DSG-1, and DSG-3) or compound 20 (ZO-1 and JAM-A) in tris(hydroxymethyl)aminomethane-hydrochloric acid buffer at 37 °C for 1 hour before substrate addition. Proteins were separated by SDS-PAGE and analyzed by Coomassie (FLG) or western blot (E-cadherin). Blots were imaged on LI-COR Odyssey Fc system at 700 nm (LI-COR Biosciences, Lincoln, NE).

Immunocytochemistry

HaCaTs at confluence were serum-starved for 4 hours, then exposed to 0–200 nM GzmB for 8 hours. Immunocytochemistry was performed as previously reported (Supplementary Table S4) (Turner et al., 2019). Images were captured by Zeiss AxioObserver Z.1 laser scanning confocal microscope, using Zen Software (Zeiss, Jena, Germany).

TEWL

Measurements of TEWL were obtained with a calibrated VapoMeter evaporimeter (Delfin Technologies, Kuopio, Finland) following the manufacturer's instructions.

Detection of sE-cadherin fragments

sE-cadherin fragments were detected in 20-fold concentrated (Amacon spin columns, Millipore, Burlington, MA) HaCaT culture supernatants by western blot. E-Cadherin antibodies to N-terminal region identified soluble fragments (Abcam, Cambridge, MA).

OXA-induced murine model of dermatitis

The OXA-dermatitis model was reported previously (Chapman et al., 1986; Kitagaki et al., 1997; Martin, 2013; Park et al., 2011; Webb et al., 1998; Zachariassen et al., 2017). *GzmB*^{+/-} mice (C57BL/6 background) were from Jackson Laboratories (B6;129S2-Gzmbtm1Ley/J, Bar Harbor, ME) and bred to *GzmB*^{-/-} mice at our animal facility. C57BL/6 WT mice were littermates from *GzmB*^{-/-} breeding. Animals (8–11 weeks old), maintained at less than six generations before back crossing, were fed and watered ad libitum and maintained on a 12-hour light and/or dark cycle. Six mice were included per treatment group with female mice included in the

study. VTI-1002 (3.6 mg/ml) formulated using propriety vehicle was applied (50 μ l) to one ear, with the other ear receiving vehicle only. Mice were treated with the topical VTI-1002 formulation once daily from day 0 to day 26. On the days when OXA and VTI-1002 were both applied, VTI-1002 was administered 30 minutes before OXA, allowing sufficient time for the topical gel to be absorbed. We adapted the Severity Scoring AD and the Three-Item Severity developed to assess dermatitis severity (Supplementary Table S2) (Kunz et al., 1997; Oranje, 2011). Scores were determined by multiple independent and blinded assessors ($n = 3$).

IHC, immunofluorescence, and TBO stain

IHC, immunofluorescence (Shen et al., 2012), and TBO staining (Parkinson et al., 2015) were performed as previously reported (Supplementary Table S4). Images were captured using Zeiss AxioObserver Z.1 laser scanning confocal microscope, using Zen Software (Zeiss).

Morphometric analysis

Percent lesion was calculated as percentage of the whole ear section without intact epidermis. IHC and/or immunofluorescence intensity was calculated: GzmB as fluorescence per total ear area; E-cadherin, DSG-1 and DSG-3, and ZO-1 as the intensity in epidermis per total area; FLG as the intensity in SC per total SC area; and mast cells as total number of TBO⁺ cells in dermis per unit area. Slides were blinded before analysis ($n \geq 4$ per group). TBO and/or GzmB colocalization were determined by overlaying images from individual tissue sections after sequential staining using Photoshop (Adobe Systems, San Jose, CA).

IL-18 ELISA

IL-18 was quantified in mouse tissue extracts ($n = 4$ per group) by ELISA as per kit instructions (ab216165, Abcam, Cambridge, MA).

Skin cleavage assay

Fresh healthy human skin was trimmed into 1×4 mm sections with the adipose tissue layer removed. Skin samples were incubated in 300 μ l PBS, 200 nM GzmB, or 200 nM GzmB preincubated for 1 hour at 37°C with 200 μ M VTI-1002 for 12 hours at 37°C. Following incubation, samples were fixed in 10% (v/v) buffered formalin, paraffin-embedded, and sectioned for E-cadherin and FLG IHC. Only the outermost sections of the skin were used for staining.

Statistical analysis

Statistical differences in experiments were determined using Student's *t*-test (two-sided, nonpaired) or ANOVA, with Bonferroni post-test used for group comparison analyses, and $P < 0.05$ was considered significant. For data not following a normal distribution, Mann–Whitney *U* test was performed. Cell culture data represent results from three independent experiments. Error bars were SEM except the in vitro experiments, which were SD.

Study approvals

Skin from patients with AD was obtained from McMaster University, Ontario, Canada, (see Supplementary Table S5 for inclusion and/or exclusion criteria) with approval (#2317). Skin from patients with psoriasis and skin from healthy volunteers was obtained from the University of British Columbia, Canada, and Vancouver General Hospital Burns Clinic, British Columbia, Canada, with approval (H15-03345 and H12-00540). Written informed consent was obtained for each sample. Animal studies were approved by the Animal Experimentation Committee of the University of British Columbia, Canada (#A17-0232).

Data availability statement

No datasets were generated during this study.

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CONFLICT OF INTEREST

DJG is cofounder and chief scientific officer of viDA Therapeutics. The remaining authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: DJG, CTT; Data Curation: RS, HL, GMG; Formal Analysis: CTT, MRZ, SH, DJG; Funding Acquisition: DJG, CTT, MRZ; Investigation: CTT, KCR, MRZ, SS, SH, CW, HZ, YS; Writing - Original Draft Preparation: CTT, DJG; Writing - Review and Editing: CTT, KCR, MRZ, SS, SH, CW, HZ, YS, RS, HL, GMG, DJG

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <https://doi.org/10.1016/j.jid.2020.05.095>.

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