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TWEAK/Fn14 Signaling Involvement in the Pathogenesis of Cutaneous Disease in the MRL/lpr Model of Spontaneous Lupus

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Abstract

TNF-like weak inducer of apoptosis (TWEAK, TNFSF12) and its sole receptor Fn14, belonging to the TNF ligand and receptor superfamilies respectively, are involved in cell survival and cytokine production. The role of TWEAK/Fn14 interactions in the pathogenesis of cutaneous lupus has not been explored. TWEAK treatment of murine PAM212 keratinocytes stimulated the secretion of RANTES via Fn14, and promoted apoptosis. Parthenolide, but not wortmanin or the MAPK inhibitor PD98059, significantly decreased production of RANTES, indicating that this effect of TWEAK is mediated via NF-κB signaling. Ultraviolet-B irradiation significantly upregulated the expression of Fn14 on keratinocytes in vitro and in vivo, and increased RANTES production. MRL/lpr Fn14 knockout lupus mice were compared with MRL/lpr Fn14 wild-type mice to evaluate for any possible differences in the severity of cutaneous lesions and the presence of infiltrating immune cells. MRL/lpr Fn14 knockout mice had markedly attenuated cutaneous disease as compared to their Fn14 wild-type littermates, as evidenced by the well maintained architecture of the skin and significantly decreased skin infiltration of T cells and macrophages. Our data strongly implicate TWEAK/Fn14 signaling in the pathogenesis of the cutaneous

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CONFLICT OF INTEREST

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manifestations in the MRL/lpr model of spontaneous lupus, and suggest a possible target for therapeutic intervention.

INTRODUCTION

SLE is a multisystem autoimmune disease with a complex and yet to be fully elucidated etiology (Rahman and Isenberg 2008; Tsokos 2011). The skin is very commonly affected, with approximately 2/3 of patients developing cutaneous manifestations (Mikita et al, 2011). Additionally, in 25% of patients skin involvement can appear before the onset of systemic symptoms (Winkelmann et al, 2013). The morphology of cutaneous lupus can vary significantly, from bullous lesions in an acute form to atrophic, scarring dyspigmented plaques in the chronic type (Lin et al, 2007). Of the current approved therapies, none are approved explicitly for the treatment of cutaneous lupus (Winkelmann et al, 2013).

Animal models have been instrumental in investigating and understanding many crucial aspects of human SLE. Several different mouse strains, both transgenic and spontaneous, are employed in the study of cutaneous lupus. The most commonly used model to study lupus skin disease is the MRL/lpr inbred strain (Ghoreishi and Dutz 2009). MRL/lpr mice are homozygous for the lymphoproliferation spontaneous mutation (Fas^{lpr} – TNF receptor superfamily member 6; also known as CD95). Fas plays a role in thymic selection and T-cell survival; the protein is not found on lymphocytes of MRL/lpr mice, leading to defects in apoptosis (Drappa et al, 1993). MRL/lpr mice spontaneously develop disease that very closely mimics human systemic lupus erythematosus, with multiple organ systems affected. Both T and B cells aberrantly proliferate, resulting in glomerulonephritis, lymphadenopathy, arthritis, and skin disease, accompanied by high titers of anti-nuclear antibodies and immune-complex deposition (Cohen and Einsenberg 1991). Features of cutaneous disease in MRL/lpr mice include spontaneous onset of skin lesions similar histopathologically to human lupus, overlap in cytokine expression, and sensitivity to ultraviolet (UV) irradiation (Menke et al, 2008).

Several members of the TNF/TNF receptor superfamily are instrumental in the pathogenesis of lupus (Ohl and Tenbrock 2011), including the TNF, BLyS, and CD40L pathways (Alaaeddine et al, 2012; Vincent et al, 2012; Zhang et al, 2012). Another TNF superfamily cytokine more recently recognized to play a role in SLE is TWEAK (Campbell et al, 2006; Winkles 2008). TWEAK functions primarily as a soluble cytokine, whose only known signaling receptor is Fn14, a Type I transmembrane protein (Wiley and Winkles 2003). Notably, the expression of this pathway is increased in settings of tissue injury and disease (Burkly et al, 2007). Furthermore, TWEAK/Fn14 signaling significantly contributes to pathogenesis in murine models of arthritis, multiple sclerosis, and inflammatory bowel disease. Mechanistic studies have elucidated TWEAK/Fn14 pathway regulation of multiple disease-driving mechanisms, including cell proliferation, cell death, angiogenesis, inflammation and fibrosis, and tissue repair (Zheng and Burkly 2008; Burkly et al, 2011).

The role of TWEAK/Fn14 in skin diseases has been scarcely studied. Fn14 expression is increased in atopic and seborrheic eczema. However, TWEAK expression was decreased in these lesions, and circulating TWEAK levels were not elevated (Chen et al, 2011).

Similarly, Peternel found decreased expression of TWEAK in a variety of inflammatory and neoplastic skin diseases, including psoriasis, lichen planus, actinic keratosis, basal cell carcinoma, and keratoacanthoma (Peternel et al, 2011). In contrast, increased TWEAK was found in human atopic dermatitis lesions (Zimmermann et al, 2011), while Fn14 was elevated in >90% of melanomas (Zhou et al, 2013). Finally, TWEAK and Fn14 were highly expressed in urticarial vasculitis (Li et al, 2013). TWEAK may contribute to skin disease via the major biological processes TWEAK affects, including cell death and promotion of inflammatory cytokines. Thus, we hypothesized that TWEAK is involved in the pathogenesis of cutaneous disease in SLE, a theory that has not been previously investigated.

The primary purpose of this study was then to evaluate a possible role of the TWEAK/Fn14 axis in the development of skin disease in the MRL/lpr murine model of human cutaneous lupus. Interestingly, UV exposure is a major trigger of cutaneous lesions and systemic disease in lupus patients. Similar to TWEAK/Fn14 signaling, UV irradiation promotes chemokine secretion and apoptosis (Kuhn et al, 2014). Therefore, we also explored the possibility that UV irradiation upregulates the expression of Fn14 and thereby potentiates the effects of the TWEAK/Fn14 signaling pathway.

RESULTS

PAM212 keratinocytes and WEHI164 fibroblasts express Fn14

To determine whether murine skin cells can be TWEAK responsive, we assessed for Fn14 receptor expression. Fn14 expression in the murine keratinocyte PAM212 cell line was comparable to that of mesangial cells, which are known to express Fn14 (Figure 1a). By flow cytometry, we confirmed that PAM212 keratinocytes have Fn14 on their cell surface (Figure 1b). Primary keratinocytes were analyzed as well, and were also found to express Fn14 (Figure 1b). Similarly, we found that WEHI164 murine dermal fibroblasts express Fn14 by RT-PCR (Figure 1a) and flow cytometry (Figure 1b), consistent with the ability of human fibroblasts to respond to TWEAK (Chicheportiche et al, 2002).

TWEAK promotes RANTES production in keratinocytes

Depending on the particular cell type, TWEAK can induce cytokines including IL-6, IL-8, GM-CSF, MCP-1, and RANTES (Burkly et al, 2007). To determine which are the inflammatory mediators most relevant to the effects of TWEAK on keratinocytes, we used a multiplex array. RANTES was one of the most highly expressed cytokines in this assay; concentrations were ~4-fold higher in supernatants from Fc-TWEAK treated cells (not shown). We measured RANTES levels by ELISA in conditioned medium from PAM212 keratinocytes treated with Fc-TWEAK, and confirmed that Fc-TWEAK induces RANTES production (Figure 1c). Production of RANTES is at least partially mediated by NF-κB signaling, as inhibition of this pathway with parthenolide significantly decreased production of RANTES (Figure 1d). In contrast, the PI3K inhibitor wortmannin or the MAPK inhibitor PD98059 did not affect RANTES production (Figure 1d).

TWEAK induces keratinocyte apoptosis

We utilized annexin-V staining to assess keratinocyte apoptosis induced by TWEAK. We found that ~25% of keratinocytes treated with Fc-TWEAK were annexin-V+; this level of apoptosis was not seen with untreated or control treated cells (Figure 1e). Previously we determined that the proapoptotic effects of TWEAK are enhanced when used in combination with IFN-γ (Campbell et al, 2006). Indeed, there was an increase in annexin-V+ cells following treatment with a combination of Fc-TWEAK+IFN-γ (Figure 1e). We also studied TWEAK-induced apoptosis in primary murine keratinocytes and found a similar trend to that observed in PAM212 (not shown).

UVB irradiation upregulates Fn14

Fn14 is consistently expressed at low levels in normal tissues, but is upregulated following a variety of tissue insults (Burkly et al, 2011). To assess the effect of ultraviolet-B (UVB) light on Fn14 expression, PAM212 keratinocytes were exposed to UVB and the expression of Fn14 measured using RT-PCR and flow cytometry. RT-PCR revealed a ~8-fold increase of Fn14 expression in UVB-treated cells (Figure 2a). With UVB, and enhanced with TWEAK co-treatment, increased Fn14 expression was similarly observed when irradiated cells were analyzed by flow cytometry, suggesting that the receptor is prominently upregulated on a cellular subset following irradiation (Figure 2b). Importantly, upregulation of Fn14 was also seen in vivo, with an increase in dermal Fn14 staining following UVB irradiation of MRL/lpr mice (not shown).

UVB in combination with TWEAK increases RANTES and apoptosis in keratinocytes

Since UVB is involved in the pathogenesis of cutaneous lupus through both proapoptotic and proinflammatory effects, we wanted to assess if UV enhances the effect of TWEAK on skin cells. We found that a combination of UVB+Fc-TWEAK led to a significant increase in RANTES production by PAM212 cells (Figure 2c). Confirming that this heightened effect observed with UVB+Fc-TWEAK in combination was not limited to RANTES, we examined the expression of CSF-1 (a cytokine implicated in cutaneous lupus (Menke et al, 2008)) and found a similar enhancement following exposure to both stimuli (not shown). The heightened effect of UVB+Fc-TWEAK in induction of RANTES was prevented by an anti-TWEAK antibody (Figure 2d), indicating that the RANTES inducing effects of UV on keratinocytes are primarily mediated by Fn14 upregulation and sensitization of the cells to the effects of TWEAK, rather than ligand independent signaling (Winkles 2008). Cells treated with UVB+anti-TWEAK antibody alone, but without TWEAK, did not produce RANTES (not shown). Furthermore, we found that keratinocytes treated with UVB+Fc-TWEAK showed a significantly increased apoptotic response (Figure 3a, 3b). A similar result was found when comparing UVB+Fc-TWEAK versus UVB alone (Figure 3a, 3b). Taken together, these results point to a shared effect of UVB and TWEAK that can drive the production of RANTES and promote keratinocyte apoptosis.

MRL/lpr mice lacking Fn14 have attenuated cutaneous disease

MRL/lpr mice spontaneously develop severe cutaneous lesions that share multiple features with human chronic cutaneous lupus, including erythematous, hyperkeratotic, and often

eroded plaques macroscopically, and interface dermatitis with peri-adnexal lymphocytic infiltrate and follicular plugging microscopically (Furukawa et al, 1996). To evaluate the role of TWEAK/Fn14 in the pathogenesis of cutaneous lupus, we generated and studied Fn14 knockout (KO) mice on the MRL/lpr background. MRL/lpr Fn14 wild-type (WT) and MRL/lpr Fn14 KO mice were scored blindly to assess severity of skin disease. Male and female mice were initially scored separately; since the differences in skin scores at this age were not significant, mice from both genders were combined. MRL/lpr Fn14 KO mice had significantly attenuated skin disease as compared to MRL/lpr Fn14 WT mice (Figure 4a).

When analyzed histologically, we found that MRL/lpr Fn14 WT mice showed characteristic cutaneous lupus lesions, including an acanthotic epidermis, follicular plugging, interface dermatitis, and in some cases, a lobular panniculitis with evidence of hyalanizing necrosis (Figure 4b). In contrast, MRL/lpr Fn14 KO mice showed relatively normal skin architecture, with no thickening of the epidermis or involvement of the dermo-epidermal junction (Figure 4b). Detailed macroscopic scoring revealed that MRL/lpr Fn14 WT mice had more erythema, thickness, and scaling of the skin as compared to MRL/lpr Fn14 KO mice, particularly in both the trunk (Figure 4c) and head regions. No significant difference in the scores for alopecia was seen between the MRL/lpr Fn14 WT (0.75±0.16) and the MRL/lpr Fn14 KO (0.5+0.28) strains ($p=0.43$).

Immunohistochemistry confirmed more active inflammation in the skin of MRL/lpr Fn14 WT mice, with greater infiltration of CD3+ T cells concentrated perifollicularly as well as IBA-1+ macrophages (Figure 5a and 5b). In contrast, MRL/lpr Fn14 KO mice had very few infiltrating cells, none of which were concentrated in particular areas of the skin. Skin from MRL/lpr Fn14 WT but not KO mice stained positive in a TUNEL assay, indicating that cell death by apoptosis is more prominent in the skin of Fn14 WT lupus mice (Figure 5c).

Finally, to determine the possible relevance of the TWEAK/Fn14 pathway to human disease, in a preliminary study we stained skin biopsies from lupus patients for Fn14. As seen in Figure 6, 3 of 3 lesional lupus skin biopsies demonstrated prominent Fn14 staining, particularly in the dermis. Interestingly, in some biopsies of non-lesional skin, epidermal Fn14 staining was present as well (Figure 6).

DISCUSSION

We found that TWEAK signaling via Fn14 plays an important role in the pathogenesis of skin disease in the MRL/lpr lupus strain. Our studies confirm the presence of the Fn14 receptor on both transformed and primary murine keratinocytes, and demonstrate that TWEAK induces apoptosis and RANTES production. Adding UVB further increases the levels of Fn14 expression, keratinocyte apoptosis, and RANTES when compared to TWEAK or UVB alone. Interestingly, not all keratinocytes upregulated Fn14 with UVB, perhaps due to cell-cycle related differences in sensitivity to irradiation. In vivo, MRL/lpr Fn14 KO mice have significantly attenuated skin disease and markedly fewer skin infiltrating macrophages and T cells. This protection is likely to be mediated by abrogated TWEAK/Fn14 mediated stimulation of keratinocyte RANTES production and apoptosis.

Several studies have demonstrated the involvement of TWEAK in the pathogenesis of autoimmune disease models, including arthritis, EAE, and colitis (Desplat-Jégo et al, 2005; Kamata et al, 2006). More recent studies have shown that the TWEAK/Fn14 axis contributes to end organ pathology in systemic lupus; TWEAK/Fn14 pathway inhibition attenuated lupus associated nephritis (Michaelson et al, 2012; Xia et al, 2014) and neuropsychiatric disease (Wen et al, 2013). Our current report is unique in that it supports a previously unrecognized involvement of TWEAK/Fn14, possibly in conjunction with UVB irradiation, in the pathogenesis of cutaneous lupus manifestations.

The detrimental effects of UV on keratinocytes are comparable in mice and humans. In vitro, keratinocytes from MRL/lpr mice and humans are susceptible to UV toxicity (Lin et al, 2007). In the MRL/lpr strain in vivo, UVB activates and recruits macrophages, which then can release mediators that induce keratinocyte apoptosis and exacerbate skin disease (Menke et al, 2008). Similarly, UV is a well-known trigger for skin disease in human lupus patients. Skin is routinely exposed to UVA and UVB, both of which can elicit the formation of cyclobutane pyrimidine dimers and reactive oxygen species which can damage DNA. Importantly, keratinocytes in particular are often the main target of UV-induced inflammation, DNA damage, and apoptosis because of their superficial localization within the epidermis (Matsuda et al, 2010). UV can induce apoptosis and necrosis, which can initiate immune responses through uptake of apoptotic blebs by dendritic cells (Furukawa 2003; Schulze et al, 2008). Defective clearance and/or an excess supply of apoptotic debris can provide an abundant supply of lupus-related autoantigens, thus promoting the generation of autoantibodies and leading to immune-complex formation, deposition at the dermal-epidermal junction, and complement activation (Oke and Wahren-Herlenius 2013).

TWEAK induces the production of RANTES, a T cell chemoattractant important in the pathogenesis of psoriasis (Fukuoka et al, 1998) and atopic dermatitis (Kaburagi et al,. 2001). Interestingly, Van Nguyen recently showed intense RANTES staining in the skin of lupus patients with cutaneous involvement, particularly epidermal keratinocytes (Van Nguyen et al, 2011). UV irradiation can lead to increased production of chemokines/cytokines by keratinocytes and other cell types in the skin, which also promotes local inflammation (Lee et al, 2013). Regarding the production of RANTES by human keratinocytes induced by UV irradiation, studies have been conflicting. Arakawa found that UVB irradiation alone of human epidermal keratinocytes could not induce the production of RANTES; only when cells were treated with TNF or IFN-γ following irradiation did production of RANTES become evident (Arakawa, 2006). In contrast, Van Nguyen reported RANTES induction by UVB in a time and dose dependent manner (Van Nguyen et al, 2011).

Our results are consistent with previous studies showing that human keratinocyte-derived cells, HaCaT, have increased apoptosis after TWEAK exposure. This same study also looked at Fn14+ primary cells from patients with atopic dermatitis and psoriasis; these cells did not undergo apoptosis when treated with TWEAK alone, but became more sensitive to the apoptotic effect of TWEAK when pre-treated with cyclohexamide or in combination with TNF (Zimmermann et al, 2011). Similarly, Fn14+ human foreskin keratinocytes showed a dose dependent apoptotic response to TWEAK (Sabour Alaoui et al, 2012).

We clearly demonstrate here apoptosis and production of RANTES following TWEAK treatment of murine keratinocytes, which had not been previously described (Jin et al, 2004). This is an important finding, as it is of direct relevance to the pathogenesis of cutaneous disease in MRL/lpr mice. Interestingly, TWEAK signaling can proceed via multiple intracellular signaling pathways include NF-κB, MAPK (via ERK, JNK, or p38), and PI3K/AKT (Burkly 2011, Winkles 2008). Our studies showed that RANTES production was only blocked by parthenolide, but not inhbitors of MAPK or PI3K, indicating that NF-κB signaling is the pathway relevant for RANTES production via Fn14 in keratinocytes. Another unique observation was that exposure to UVB+TWEAK lead to a synergistic increase in RANTES, likely through sensitization of the keratinocytes to the inflammatory effects of TWEAK mediated by the increased expression of Fn14 following UVB.

Our findings in this study strongly suggest a previously unrecognized pathway in the pathogenesis of chronic cutaneous lupus erythematosus. We propose a model in which TWEAK stimulation of keratinocytes leads to the production of RANTES, a known chemotactic protein for T cells and other inflammatory cells. Exposure to UVB in addition to TWEAK may further exacerbate the production of RANTES (and perhaps other cytokines as well), creating an inflammatory local environment and further attracting infiltrating cells. TWEAK, as does UV irradiation, also leads to apoptosis of keratinocytes, which can further contribute to the development of the cutaneous lesions.

The pathogenesis of inflammatory skin disease in SLE is admittedly complex, and most likely involves multiple cytokines and several cellular pathways, not all of which are directly related to, or influenced by, TWEAK/Fn14 interactions. Nevertheless, the protection of MRL/lpr Fn14 KO mice from the macroscopic and microscopic manifestations of lupus-like skin disease in this model is highly significant, and suggests that blockade of even a single cytokine may be beneficial for this particular disease manifestation. Furthermore, Fn14 upregulation was present in human lupus skin. It remains to be determined in future studies how TWEAK combines with other central pathogenic pathways, such as UVB, in triggering cutaneous inflammation in the intact animal as well.

In summary, we have demonstrated that TWEAK and UVB, alone and in combination, lead to inflammation and apoptosis in murine keratinocytes. Furthermore, MRL/lpr Fn14 KO mice have significantly attenuated cutaneous disease with reduced infiltration of T cells and macrophages. The results from the in vitro studies and in vivo correlative studies suggest that Fn14 deficiency is protective due to reduced RANTES and keratinocyte apoptosis. We propose that the TWEAK/Fn14 axis contributes to the pathogenesis of cutaneous lupus, and that this signaling pathway represents a potential therapeutic target for biological agents.

MATERIALS AND METHODS

Cells and reagents

PAM212, a BALB/c derived keratinocyte cell line (Tron et al, 1990), and WEHI164, a skin fibroblast cell line established from a BALB/c-derived fibrosarcoma, were grown in 10% DMEM, and maintained at 37°C/5% CO_2. Isolation and culture of primary keratinocytes was performed essentially as described (Jensen et al, 2013). For stimulation assays, cells

were incubated with murine Fc-TWEAK (Wu et al, 2013), or an IgG2a isotype control P1.17 (ATCC).

RNA isolation and RT-PCR

RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA), and reverse transcription was performed using the Superscript First-Strand System (Life Technologies, Norwalk, CT). Real-time PCR was performed in triplicate by the SYBR green method. The gene of interest was normalized to the average of two housekeeping genes (GAPDH, tubulin), and fold changes were determined using the delta delta CT method.

Flow cytometry for detection of cell surface Fn14 expression

PAM212 keratinocytes and WEHI164 fibroblasts were detached with 0.25% Trypsin-EDTA, then washed and resuspended in FACS buffer. A total of 2.5×10^5 cells was incubated with an Fn14-specific Fab'$_2$ (derived from P4A8) (Michaelson et al, 2011) or a control Fab'$_2$ MOPC-21 for 30 minutes. Cells were washed three times, and flow cytometry performed on a FACSCalibur instrument. Primary keratinocytes were similarly detached, and incubated with 0.5 μg/ml Fc-block for 5 minutes at 4°C. ITEM-4 (Santa Cruz Biotechnology, Dallas, TX) or control murine IgG2b was added for 60 minutes at 4°C. Next, the cells were washed in FACS buffer, and incubated with biotinylated goat anti-mouse IgG2b for 30 minutes at 4°C followed by APC-streptavidin for 30 minutes at 4°C.

Apoptosis

Keratinocytes (3×10^4) were allowed to adhere overnight at 37°C/5% CO_2. Fc-TWEAK, control Ig, or Fc-TWEAK+IFN-γ were added to each well for 48 hours. Following treatment, cells were harvested and resuspended in annexin binding buffer in 2% FBS. Annexin-FITC was added to each sample and incubated for 15 minutes on ice. 7-AAD was then added and samples were immediately processed on a FACSCalibur or LSRII instrument. In this assay, early apoptotic cells are annexin+ and 7-AAD-, while late apoptotic cells stain positive both for annexin and 7-AAD.

Cytokine array

PAM212 cells were treated with Fc-TWEAK or control Ig for 48 hours. Supernatants were collected, and incubated with pre-coated membranes for cytokine detection (AAM-CYT-1) (RayBiotech, Norcross, GA).

RANTES ELISA

PAM212 cells (5×10^4) were seeded in multiwell plates and allowed to adhere overnight. The following day media was removed, and the cells were washed and treated with Fc-TWEAK, control Ig, IFN-γ, Fc-TWEAK+IFN-γ, or control Ig+IFN-γ. Supernatants were analyzed using a RANTES ELISA Kit (R&D Systems, Minneapolis, MN).

UVB irradiation

PAM212 cells were exposed to UVB light using a 302 nm UVM-57 lamp (UVP, Upland, CA), and either left in media alone, or treated with Fc-TWEAK or control Ig for 48 hours.

RANTES concentrations were measured by ELISA, or the cells were harvested and analyzed for apoptosis as described above. For quantification of gene expression, cells were harvested after 24 hours, and RNA was isolated for RT-PCR.

Cell signaling

PAM212 cells (5×10^4) were washed with PBS and pre-treated for two hours with 10 μM parthenolide (Santa Cruz), wortmannin (Tocris Biosciences, Minneapolis, MN), or PD98059 (Cell Signaling, Beverly, MA), followed by Fc-TWEAK or control Ig for 48 hours. Supernatants were analyzed by RANTES ELISA.

Animals

MRL/lpr Fn14 KO (9^{th} generation) and WT littermates were maintained at Einstein (Jakubowski et al, 2005). MRL/MpJ mice were from Jackson Laboratories (Bar Harbor, ME). All animal study protocols were approved by the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine.

Scoring of skin lesions

Mice were scored blindly by trained observers every two weeks from 26 to 54 weeks of age, and an average score recorded for each mouse. Plaque characteristics in multiple body regions were given numerical scores, and were further adjusted for the extent of involvement and body surface area covered (Supplemental Figure 1). The scoring system was designed combining features of the Psoriasis Area and Severity Index (PASI) and the Cutaneous Lupus Area and Severity Index (CLASI) scoring systems, with the maximum score being 72 (Albrecht et al, 2005; Feldman and Krueger 2005).

Histopathology and immunohistochemistry

Tissue samples were immersed in 10% buffered formalin for 48 hours at 4°C. Samples were then transferred to 70% ethanol and paraffin embedded, and stained using hematoxylin and eosin (H+E). For immunohistochemical staining, reagents included antibodies to CD3, IBA-1, and Fn14. Following the addition of substrate solution, slides were rinsed in ddH20, stained in Mayer's hematoxylin, washed, air-dried, and mounted with Permount (Fisher, Pittsburgh, PA).

Human skin biopsies were obtained following written informed consent, with the protocol approved by the IRBs at NYU and the Albert Einstein College of Medicine. For paraffin slide staining, heat-induced antigen retrieval was done using a pH 6.0 citrate buffer for 10 minutes. After cooling, slides were washed in PBS and subsequently blocked in dual endogenous enzyme block for 10 minutes. Following two washes in PBS-Tween, slides were incubated with a rabbit anti-human antibody to Fn14 (Cell Signaling, Beverly, MA) at a dilution of 1:100 in 1% PBS/BSA overnight at 4°C. Slides were washed in PBS-Tween and incubated with biotinylated anti-rabbit secondary antibody (2 hours at RT) followed by streptavidin-HRP (30 minutes at RT). Color was developed using DAB substrate, and slides were counterstained using hematoxylin.

Analysis

The results shown are mean+SE. Values of $p<0.05$ were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TWEAK	TNF-like weak inducer of apotosis
KO	knockout
WT	wild-type
UV	ultraviolet
UVB	ultraviolet B

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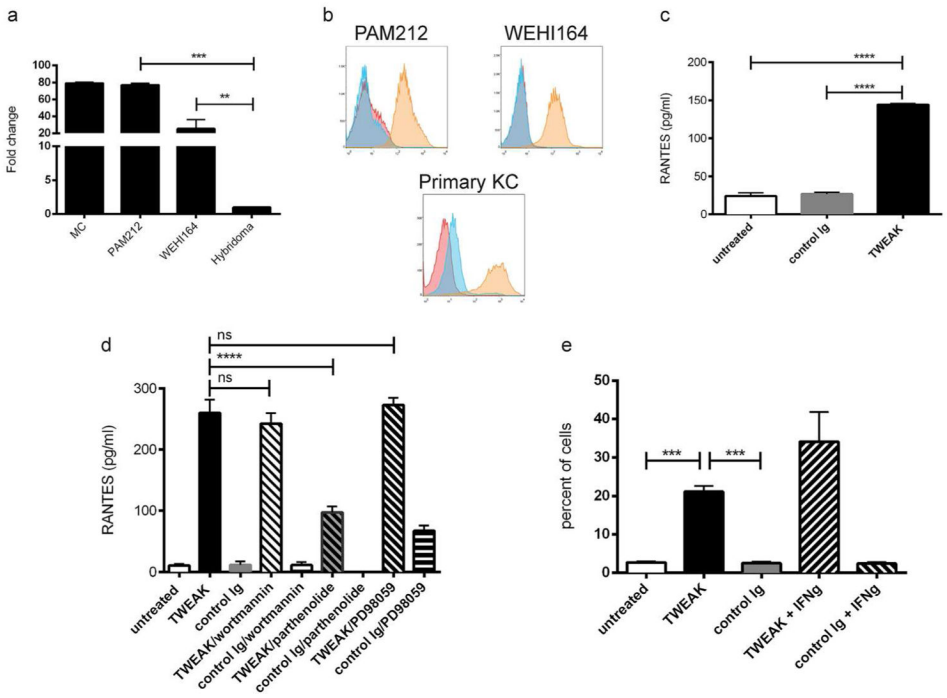

Figure 1. Fn14 expression and RANTES production in skin cells

(a) RT-PCR for Fn14 expression, with fold change calculated versus an irrelevant hybridoma set at 1. (b) Fn14 expression by flow cytometry (red=unstained, blue=control, orange=Fn14). (c) PAM212 keratinocytes were untreated, or incubated with TWEAK or control Ig (0.1 μg/ml) for 48 hours. (d) PAM212 keratinocytes were pretreated with wortmanin, parthenolide, or PD98059, followed by Fc-TWEAK or control Ig for 48 hours. (e) PAM212 were treated for 48 hours (1 μg/ml Fc-TWEAK, 1 μg/ml control Ig, 1 μg/ml Fc-TWEAK+80 IU/ml IFN-γ, and 1 μg/ml control Ig+80 IU/ml IFN-γ), and stained with annexin-V and 7-AAD. The figure shows the number of early apoptotic keratinocytes with each treatment. The figures shown are representative of 2 or 3 independent experiments. **=p<0.01, ***=p<0.001, ****=p<0.0001.

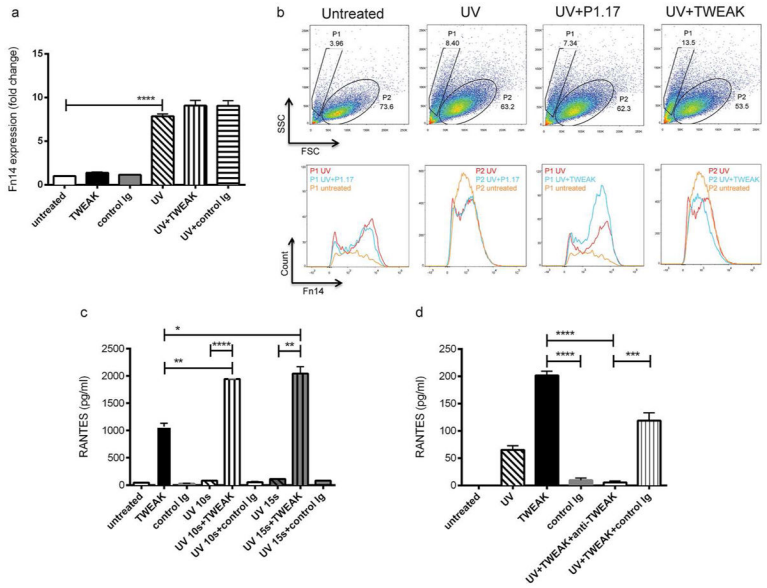

Figure 2. Effects of TWEAK and UV

(a) PAM212 were UV irradiated for 30 seconds and/or treated with TWEAK, and Fn14 measured by PCR. (b) PAM212 were treated as specified, and analyzed for Fn14 receptor expression by flow cytometry. Cells were gated in two populations, and Fn14+ cells from P1 and P2 of each group were compared. (c) Cells were UVB irradiated for 10 seconds, and treated with 1 μg/ml Fc-TWEAK or control Ig. (d) Cells were treated with Fc-TWEAK or control Ig (0.1 μg/ml) or UVB irradiated. Irradiated cells were also treated with TWEAK pre-incubated or not with an anti-TWEAK mAb. In panels (c) and (d), supernatants at 48 hours were analyzed by ELISA. The figures shown are representative of at least 2 or 3 independent experiments. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

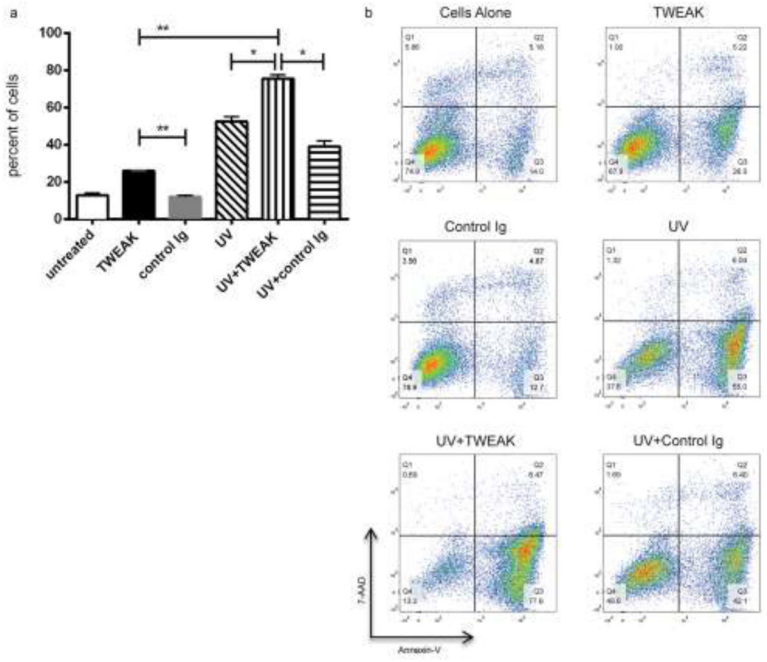

Figure 3. Induction of apoptosis by TWEAK+/−UVB

(a) PAM212 keratinocytes were subjected to UVB irradiation for 10 seconds. Cells were then treated Fc-TWEAK or control Ig (0.1 μg/ml) for 48 hours, and the degree of apoptosis analyzed by flow cytometry. The figures above are representative of 2 or 3 independent experiments. (b) Representative FACS plots of the experiment shown in (a). *=$p<0.05$, **=$p<0.01$.

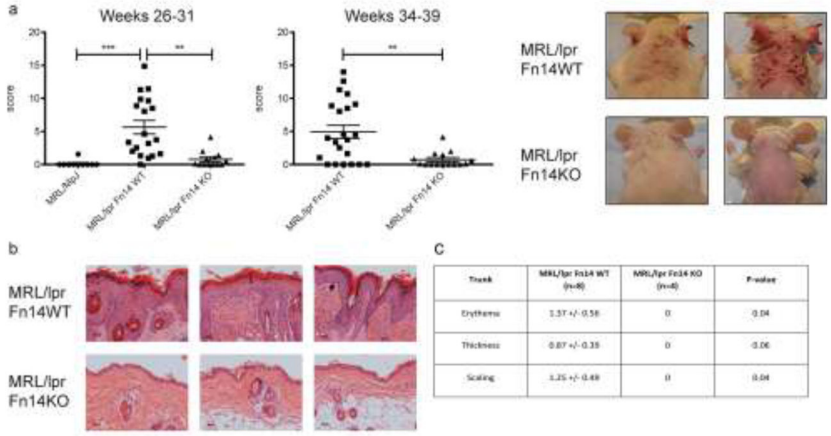

Figure 4. MRL/lpr Fn14 KO mice have attenuated skin disease

(a) Left and middle panels show skin scores from male and female mice at earlier (26–31 weeks) and later (34–39 weeks) time points (earlier: MRL/MpJ, n=10; MRL/lpr Fn14 WT, n=19; MRL/lpr Fn14 KO, n=13; later: MRL/lpr Fn14 WT, n=22; MRL/lpr Fn14 KO, n=15). Right panels show representative photographs of macroscopic skin lesions in MRL/lpr Fn14 WT (34 weeks) and MRL/lpr Fn14 KO mice (42 weeks). The left and right panels in each row of photographs depict the same mouse, before and after hair removal with Nair cream to better demonstrate the skin lesions. (b) Representative images of H&E stained sections from MRL/lpr Fn14 WT and MRL/lpr Fn14 KO mice (scale bar = 7 mm). Asterisk indicates hypergranulosis. Arrowhead indicates parakeratosis. Arrow points towards an area of acanthotic epidermis. (c) Fn14 WT mice had significantly higher scores in the trunk region. **=p<0.01, ***=p<0.001.

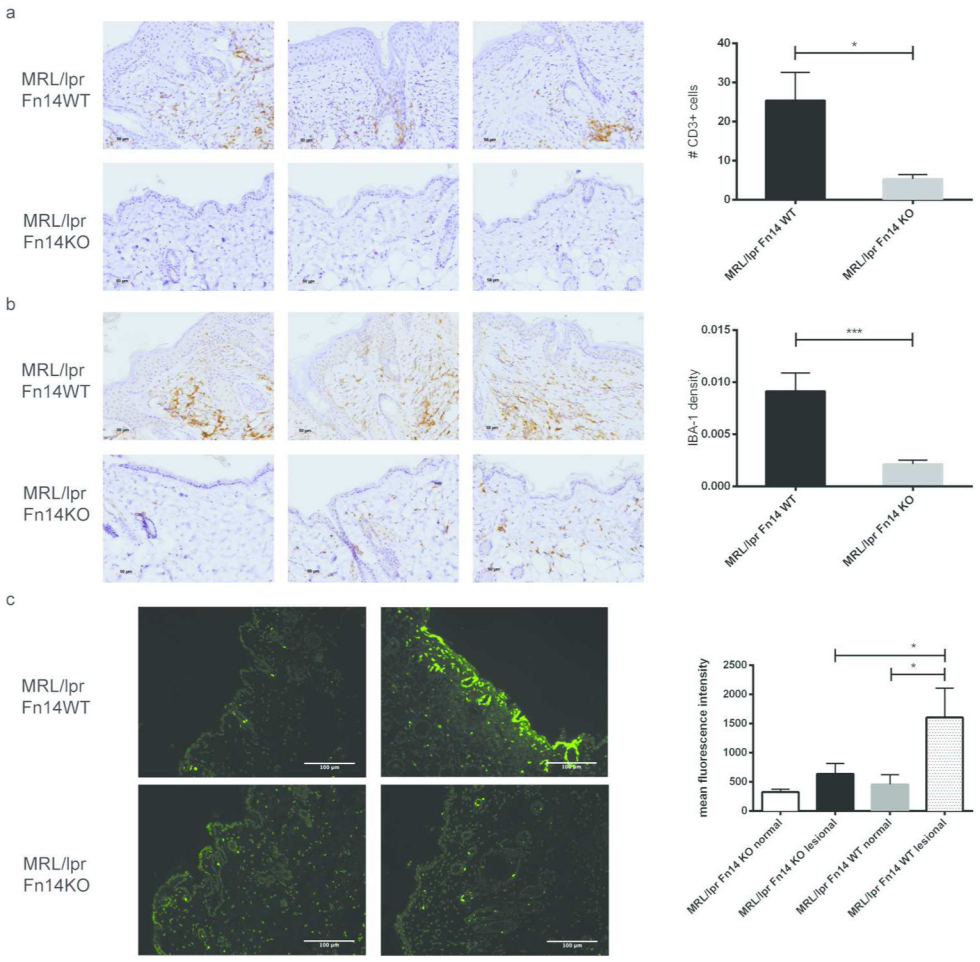

Figure 5. MRL/lpr Fn14 KO have fewer skin infiltrating cells and less epidermal apoptosis

(a) Representative images of CD3 stained sections from randomly selected MRL/lpr Fn14 WT (n=7) and Fn14 KO (n=5) mice (39 weeks) (scale bar = 7 mm). Cell counts are in the right panel. (b) Representative images of IBA-1 stained sections from randomly selected MRL/lpr Fn14 WT (n=4) and MRL/lpr Fn14 KO (n=8) mice (scale bar = 7 mm). Images were analyzed for staining density using ImageJ. (c) Lesional and unaffected skin was taken from randomly selected MRL/lpr Fn14 WT (n=6) and MRL/lpr Fn14 KO (n=6) mice. TUNEL assay was performed on paraffin embedded skin sections. Shown here is a representative image from 1 mouse in each group (scale bar = 14 mm). Fluorescence intensity was measured using ImageJ (right panel). *=$p<0.05$, ***=$p<0.001$.

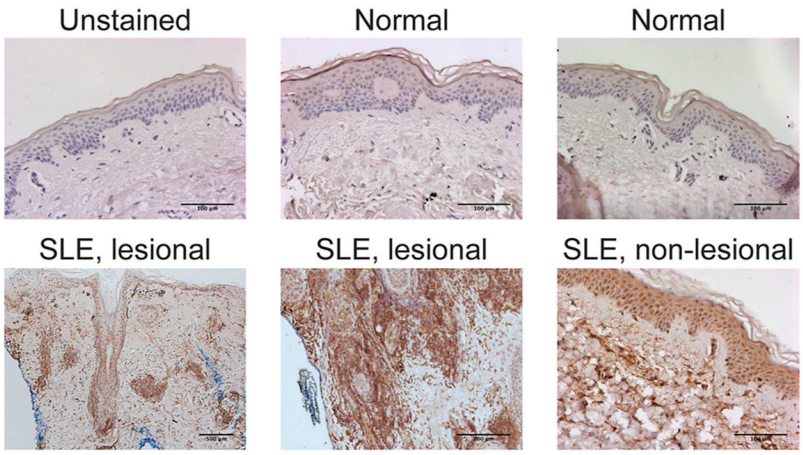

Figure 6. Human lupus skin biopsies stain positive for Fn14

Paraffin tissue sections were stained for Fn14 by immunohistochemistry, as described in the Materials and Methods. The top left panel shows the negative control (primary antibody withheld, normal skin) (scale bar = 14 mm). The top middle and right panels show stained normal skin, displaying minimal Fn14 positivity (scale bar = 14 mm). The bottom left (scale bar = 9 mm) and middle panels (scale bar = 14 mm) show two magnifications of a stained section from a lupus discoid skin lesion. There is marked dermal Fn14 staining, which is representative of the pattern seen in two additional lupus discoid skin lesion biopsies. The bottom right panel shows Fn14 staining in a non-lesional lupus skin biopsy (scale bar = 14 mm).