Exacerbation of Allergen-Induced Eczema in TLR4- and TRIF-Deficient Mice

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Exacerbation of Allergen-Induced Eczema in TLR4- and TRIF-Deficient Mice

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Despite its presence on resident skin cells, the role of TLR4 in skin diseases remains poorly understood. This is highly significant because the skin biome is rich with potential TLR4 agonists. We aimed to establish the contribution of TLR4 to atopic dermatitis and determine the mechanism by which TLR4 acts in an experimental model of atopic dermatitis. MyD88, TLR4, or Toll–IL-1R domain-containing adapter-inducing IFN-β (TRIF)–deficient and wild-type mice were epicutaneously exposed to Aspergillus fumigatus allergen over 3 wk. Impaired skin barrier function was assessed by measuring transepidermal water loss (TEWL). Skin levels of innate and adaptive genes were quantified. In an experimental model of atopic dermatitis, TEWL, allergic sensitization, and epidermal thickness were increased following cutaneous allergen exposure, and these were further enhanced in the absence of TLR4. Increased allergen-induced skin levels of innate (S100A8/A9, IL-1β, TNF-α, and CXCL2) and Th17 genes (IL-17A and IL-17F) were observed in TLR4-deficient mice compared with wild-type mice. The absence of MyD88 alleviated disease (decreased TEWL, skin thickness, proinflammatory cytokines), whereas TRIF deficiency exacerbated disease. In conclusion, signaling through the TLR4 and TRIF pathways limits skin barrier dysfunction, cutaneous allergic sensitization, and proinflammatory cytokine production. The Journal of Immunology, 2013, 191: 000–000.
the first allergen exposure. Either 200 μl sterile saline solution or *Aspergillus fumigatus* extract (Asp; Greer Laboratories, Lenior, NC), resuspended in saline solution at a concentration of 1 mg/ml, was applied to a 2 × 2-cm patch of sterile gauze. The patch was secured by Tegaderm, and the mouse was wrapped with a band aid and waterproof tape. After 6 d, the patch was removed, and 24 h later a new patch was applied for a total of three patches over a 3-wk period, as shown in Fig. 1A. Endotoxin levels in Asp were assayed using the Cambrex QC1000 assay (1 mg/ml contains 1.5–2 EU/ml or 0.3–0.4 mg endotoxin, resulting in 0.06–0.08 ng endotoxin exposure per patch).

### Measurement of transepidermal water loss

Transepidermal water loss (TEWL) was measured by using DermaLab’s instrument (DermaLab USB module; Cortex Technology, Hadsund, Denmark), as previously described (22). Briefly, TEWL was assessed over a 1-min period by placing the probe against the skin surface in the center of area exposed to the saline/allergen-soaked patch. An average of the two readings per mouse was used, and TEWL measurements were recorded as grams per meter squared per hour.

### Skin scoring system

Mice were visually assessed for excoriations, erythema, and skin thickening in the area covered by the patch. Skin thickening was scored a 0 (thickness comparable to a wild-type [WT] mouse skin), 1 (slight thickening of skin), 2 (multiple excoriations on one-third of the back), 3 (multiple excoriations on most of the back). Excoriations were scored 0 (no scratches), 1 (1–3 excoriations), 2 (multiple excoriations on the back), and 3 (significant thickening of the skin over at least two-thirds of the back).

### Mast cell staining and measurement of skin thickness

Skin samples were quenched with H2O2, blocked with 3% normal goat serum, and stained overnight at 4˚C with rat anti-mouse Ly6G (1:200; BioLegend). Plasma samples were washed and either HRP-conjugated anti-mouse IgG1 (X56; 1:1000; BD Biosciences-Pharmingen), HRP-conjugated anti-mouse IgG2c (1:400; BD Biosciences) or rat anti-mouse Ly6G (1:200; BioLegend) according to the manufacturer’s instructions (BioLegend). Cytokine levels in culture supernatants were assessed by ELISA, according to the manufacturer’s instructions, and gene expression was normalized to hypoxanthine phosphoribosyltransferase (HPRT) forward, 5'-TGCCGGAGTTTGAAGAAG-3’ and reverse, 5’-CCCCCTTGGACACACG-3’. IFN-γ forward, 5’-CAGCAACAGACGGCAAAGG-3’ and reverse, 5’-TTTCCTGCTTCAGGCTGGAF-3’, IL-4 forward, 5’-CTGAGG-GTCTCTGAGGTGTCCCG-3’ and reverse, 5’-CATTGGTGCCATGGCTTCTTAGG-3’ and reverse, 5’-AGAACATGTTGGTGTTCA-3’, IL-17A forward, 5’-ACTACCTCAAGGTCCTCACG-3’ and reverse, 5’-AGAAATCTGTTGGTGTTCA-3’, IL-17F forward, 5’-GGGAAAGACCCAGTAGTG-3’, reverse, 5’-ATGCCCCACATCACAGTG-3’, TNF-α forward, 5’-GGTGCCTGGGCGCATAGACT-3’ and reverse, 5’-CCACACGTCTTCGGTTCAC-3’, S100A8 forward, 5’-ATCCACCTGCCAACGGAATC-3’ and reverse, 5’-ATCCACCTGCCAACGGAATC-3’, S100A9 forward, 5’-GAAGGAGGACCCCTGTGACA-3’, and reverse, 5’-GTCAGGATCCT-CCATGATGT-3’, IL-6 forward, 5’-TGATGCACCTGGCACAAACA-3’ and reverse, 5’-ACAGAGGAGAAATTTCCAAGG-3’, TNF-α forward, 5’-GGGAGGCTGGCGCATAGACT-3’ and reverse, 5’-CCACACGTCTTCGGTTCAC-3’, CXCL2 forward, 5’-TCTCCCTTCAACGGCCAC-3’ and reverse, 5’-GCCTTGAGAAGTGTCCTCA-3’, CXCL2 forward, 5’-CCACACCACGGCTCACA-3’, and reverse, 5’-CCCTTTGAGATGTCCTGA-3’. ***Statistical analysis***

Reported values are expressed as mean ± SEM. Statistical analysis was performed using Prism 5 (GraphPad Software). One-way ANOVA followed by Bonferroni’s multiple comparison tests was performed on all experiments. Correlations were assessed using Spearman’s nonparametric test. Significance was set at a p value of 0.05.

### Results

#### Exacerbated skin barrier dysfunction in TLR4-deficient mice following repeated cutaneous allergen exposure

One of the cardinal features of AD is an impaired skin barrier, which is commonly demonstrated by measuring increased TEWL (22, 24). TEWL levels remained unaltered following the first *Aspergillus* patch and increased after each subsequent exposure to the allergen (Fig. 1B). Repeated epicutaneous allergen exposures result in skin inflammation and epidermal thickening as well as other clinical features of AD reflected by elevated skin scores (Fig. 1C). Asp-patch TLR4-deficient mice had markedly increased TEWL and skin scores compared with WT mice (Fig. 1B, 1C).

Because of significant baseline differences between female and male mice in the thickness of their dermis and subdermal fatty layer, we examined epidermal thickness. A 3- to 5-fold increase in epidermal thickness was observed between saline and Asp-patch mice (Fig. 1D). Epidermal thickness was significantly increased in TLR4-deficient mice compared with WT mice following the third *Aspergillus* exposure (Fig. 1D). Furthermore, a significant correlation was observed between TEWL and epidermal thickness among Asp-exposed skin samples from WT and TLR4-deficient mice (r = 0.38, p = 0.018).

#### Increased sensitization to *Aspergillus* in TLR4-deficient mice

To assess whether the observed skin barrier disruption was associated with increased allergen sensitization via the skin, plasma levels of Asp-specific IgG1, IgG2c, and IgE were determined. Three weeks of epicutaneous *Aspergillus* exposure resulted in measurable levels of Asp-specific IgG1 (Fig. 1E), but Asp-specific...
IgE or IgG2c Abs were mostly undetectable (data not shown). No significant differences in total baseline IgG1, IgG2c, or IgE levels were observed between naive TLR4-deficient and WT mice (data not shown). However, among Asp-treated mice, TLR4-deficient mice had significantly higher Asp-specific IgG1 titers compared with WT mice, indicating increased allergen sensitization (Fig. 1E).

Allergen-induced recruitment of inflammatory cells in WT and TLR4-deficient mice

Previous studies have reported that experimental AD resulting from repeated OVA exposure of tape-stripped skin is characterized not only by skin thickening, elevated OVA-specific Abs, but also by dermal accumulation of Th2 cells, eosinophils, and mast cells (25). In our experimental model, increases in inflammatory cells were observed in the dermis of Aspergillus-patched mice compared with saline-patched mice. To assess the nature of the inflammatory infiltrate, paraffin-embedded skin sections were stained for eosinophils (anti-myelin basic protein), mast cells (Leder stain), and neutrophils (anti-Ly6G). Mast cell, eosinophil, and neutrophil numbers were all significantly increased in the dermis of Asp-patched mice (Fig. 2). However, TLR4 deficiency did not significantly alter dermal eosinophil, mast cells, or neutrophil numbers following Aspergillus exposure (Fig. 2). Unlike eosinophils and mast cells, neutrophils were almost exclusively observed infiltrating the dermis below excoriations (Fig. 2C).

Increased allergen-induced IL-17A skin levels in TLR4-deficient mice

To assess the nature of the adaptive immune response, we measured Th2 (IL-4), Th1 (IFN-γ), and Th17 (IL-17A, IL-17F) cytokines in the skin beneath the patched area by real-time PCR following the second and third patches. Following the second Asp patch, IFN-γ and IL-4 mRNA skin levels remain unchanged, whereas IL-17A and IL-17F mRNA, but not IL-21 and IL-23p19 mRNA levels, were increased (Fig. 3A–C; data not shown). Despite a trend suggesting increased IL-4 generation in TLR4-deficient mice after the second Asp patch, the absence of TLR4 had no significant impact following the third Asp patch on IL-4 skin mRNA levels or IL-4 protein levels in Asp-stimulated lymph node cell cultures (Fig. 3B, 3D). Following the third Asp patch, IL-17A skin mRNA levels were increased, whereas IFN-γ were decreased in TLR4-deficient mice (Fig. 3A, 3C). TLR4 deficiency resulted in increased IL-17A protein levels in the supernatant of Asp-stimulated cells isolated from skin draining lymph nodes (Fig. 3E) consistent with the mRNA data.

Increased allergen-induced proinflammatory cytokine levels in TLR4-deficient mice

To assess the nature of the innate immune response, keratinocyte-derived genes were quantified following the second and third allergen patches. Keratinocytes are a major source of TSLP in acute and chronic lesions of AD, and TSLP has been implicated in disease pathogenesis (26–28). In our model, TSLP skin mRNA levels were increased after the second allergen patch in TLR4-deficient mice compared with WT mice, but were similar to WT mice after the third Aspergillus patch (Fig. 4A).

FIGURE 1. Impaired skin barrier function following A. fumigatus patches. (A) Experimental protocol timeline. (B) One day after each patch removal, TEWL measurements were obtained within the patched area. n = 4 mice/group; mean ± SEM; two-way ANOVA with Bonferroni posttests, ***p < 0.001. (C) Skin inflammatory score (Kruskal–Wallis ANOVA test, p < 0.0001, followed by Mann–Whitney U test between Aspergillus-exposed WT and KO mice, *p = 0.018). (D) Epidermal thickness was assessed by morphometric analysis of 3–5 pictures/mouse; representative pictures of mice taken 24 h after removal of third patch. n = 16–19/group. **p < 0.01, Bonferroni’s multiple comparison test. (E) Plasma Asp-specific IgG1 levels are expressed as OD for a specific dilution. n = 14 mice/group. *p < 0.05, one-way ANOVA with Bonferroni’s multiple comparison test.

FIGURE 2. Allergen-induced skin inflammation unaltered by TLR4 deficiency. (A) Leder-stained mast cells were counted in the dermis. (B) Dermal eosinophils were identified by immunohistochemistry for major basic protein. (C) Representative photograph of Ly6G+ neutrophils infiltrating the dermis beneath a neutrophil-rich wound. Cell counts represent the mean of 16–20 separate high power fields (40× objective). **p < 0.01, ***p < 0.001, Bonferroni’s multiple comparison test.
Among keratinocyte-derived IL-17 family members, IL-17C was significantly elevated following the second, but not third allergen patch in TLR4-deficient mice (Fig. 4B), whereas IL-25 skin levels (IL-17E) remained unchanged following Aspergillus exposure (data not shown).

In AD, keratinocytes overexpress antimicrobial peptides, including calprotectin (a heterodimer of S100A8 and S100A9). Accordingly, in our model, skin levels of S100A8 and S100A9 were elevated following Aspergillus exposure and further increased in TLR4-deficient mice compared with WT mice (Fig. 4C).

In the absence of TLR4, proinflammatory cytokines (IL-1β, IL-6, and TNF-α) were elevated following the second and third allergen patches, but only IL-1β and TNF-α were significantly increased in TLR4-deficient mice compared with WT mice (Fig. 4D–F). S100A8 and A9 mRNA skin levels were also increased in TLR4-deficient mice compared with WT mice following the second and third allergen patches (Fig. 4D and data not shown).

Neutrophil chemokines CXCL1 (KC) and CXCL2 were both increased following the second and third Asp patches, but only CXCL2 skin mRNA levels were further increased in TLR4-deficient mice compared with WT mice (Fig. 4G and data not shown). Other chemokines (CCL2, CCL11, CCL20) were not induced by Aspergillus exposure (data not shown).

MyD88 deficiency is protective, whereas TRIF deficiency exacerbates experimental AD

We next investigated which downstream pathway was involved in TLR4-mediated exacerbation of experimental AD. MyD88- and TRIF-deficient mice were subjected to the experimental AD model described in Fig. 1A. Unlike TLR4-deficient mice, the absence of MyD88 partially protected mice from allergen-induced experimental AD, as demonstrated by decreased TEWL, epidermal thickness, and epicutaneous sensitization (Fig. 5A–C). Accordingly, IL-4 and IL-17A skin mRNA levels and protein levels in Asp-stimulated draining lymph node cells were also decreased (Fig. 5D, 5E). MyD88 deficiency also impaired induction of S100A8/A9, IL-1β, IL-6, TNF-α, and CXCL2 in the skin following allergen exposure (Fig. 5F).

This was in marked contrast to the exacerbated phenotype observed in TRIF-deficient mice. Similar to TLR4-deficient mice, TRIF-deficient mice demonstrated impaired skin barrier function, as assessed by increased water loss (TEWL) following allergen exposure (Fig. 6A). Skin scores and epidermal thickness were also increased in TRIF-deficient mice, but did not reach significance (Fig. 6B, 6C). Epicutaneous sensitization was increased, as demonstrated by elevated Asp-specific IgG1 and IgG2c blood levels in TRIF- and TLR4-deficient mice (Fig. 6D). Similar to TLR4-deficient mice, total IgE levels and IL-4 skin mRNA levels were not further increased in TRIF-deficient mice compared with WT mice, and IFN-γ levels were decreased in TRIF-deficient mice (Fig. 6D, 6E). Surprisingly, skin IL-17A, which was increased in TLR4-deficient mice, was decreased in Aspergillus-exposed TRIF-deficient mice (Fig. 6E). IL-17A released by Aspergillus-stimulated lymph node cells from TRIF-deficient mice did not reach significance (Fig. 6F). In vitro restimulated lymph node cells from WT and TRIF-deficient mice released similar levels of IL-4 and IFN-γ (Fig. 6F). Consistent with skin IL-17A levels, Aspergillus-induced increases in IL-17F skin mRNA levels were impaired in TRIF-deficient mice compared with WT mice (Fig. 6G). Accordingly, skin mRNA levels of the pro-Th17 cytokine IL-6 trended lower in TRIF-deficient mice compared with WT mice (Fig. 6G). Similar to TLR4-deficient mice, TRIF-deficient mice demonstrated increased IL-1β, TNF-α, and CXCL2 skin mRNA levels compared with WT mice (Fig. 6H).

Discussion

Our data demonstrate that defective TLR4 or TRIF signaling results in disease exacerbation, as evidenced by increased skin barrier dysfunction (TEWL) and epicutaneous sensitization. Thus, sig-
naling through TLR4 protects from early inflammatory events, leading to impaired skin barrier and disease development. Mechanistically, this protective effect is most likely mediated by TRIF, not MyD88.

Our data reveal that early innate events protect from allergen-induced skin barrier dysfunction. Indeed, the increased TEWL observed after the second allergen patch in TLR4-deficient mice is associated with increases in proinflammatory cytokines (IL-1β, TNF-α). Similarly, in AD patients harboring mutations in the filaggrin gene as well as in filaggrin-deficient mice, increased skin levels of IL-1β were observed (29). Filaggrin-deficient mice also develop a local Th17 response, as evidenced by increased skin

FIGURE 5. MyD88 deficiency alleviates Aspergillus-induced skin barrier dysfunction. (A) TEWL measurements taken the day after each patch removal. n = 6–8 mice/group; mean ± SEM; two-way ANOVA with Bonferroni posttests. ***p < 0.001. (B) Epidermal thickness was assessed by morphometric analysis. (C) Asp-specific IgG1 plasma levels after the third Aspergillus patch. (D) IL-4 and IL-17A skin levels. (E) Asp-stimulated lymph node cell secretion of IL-4 and IL-17A was assessed by ELISA. (F) S100A8, IL-1β, IL-6, and CXCL2 skin mRNA levels were assessed by quantitative real-time PCR after the third patch (n = 3–6 mice/group). Representative results from two separate experiments. *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA with Bonferroni’s multiple comparison test.

FIGURE 6. TLR4 and TRIF deficiencies exacerbate experimental AD. (A) TEWL measurements after third patch (n = 9–13 mice/group from two separate experiments). (B) Skin scores were generated, as described in Materials and Methods. Skin scores and (C) epidermal thickness following the third Asp patch. (D) Plasma Asp-specific IgG1 and IgG2c levels and total IgE levels. (E) Skin mRNA levels and (F) protein levels of IL-17A, IFN-γ, and IL-4. (G) IL-17F and IL-6, and (H) IL-1β, TNF-α, and CXCL2 skin mRNA levels were assessed by quantitative real-time PCR after the third patch. n = 3–6 mice/group; representative results from two separate experiments. *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA with Bonferroni’s multiple comparison test. n.s., Not significant.
mRNA levels of IL-17A, whereas elevated skin levels of Th2 cytokines were only observed months later (5). Similarly, mice rendered deficient for either IgE, STAT6, IL-4, IL-13, mast cells, or eosinophils still develop an AD-like skin phenotype when exposed to allergen patches, indicating that none of these are essential for allergen-mediated skin inflammation (25, 30).

The Th2-promoting innate cytokine TSLP is increased in AD lesions (28). Overexpression of TSLP in keratinocytes resulted in spontaneous development of an AD-like phenotype in mice (26). In the absence of TSLP, OVA-induced increases in skin Th2 cytokines are ablated (27). Additionally, TSLP-dependent accumulation of type 2 innate lymphoid cells in AD was recently shown to contribute to disease (31). TSLP skin mRNA levels were increased in our model after the second, but not the third Aspergillus patch. Whereas we cannot exclude a role for TSLP in disease initiation, the downregulation of TSLP following the third allergen patch suggests that TSLP is not essential in disease progression.

Keratinocyte-specific overexpression of IL-17C in mice resulted in increased skin levels of IL-1β, IL-6, TNF-α, IL-17A and F, as well as the antimicrobial peptide calprotectin (S100A8/A9) (32). In our model, all of these genes were elevated after the second allergen patch, but the absence of any induction of IL-17C following the third Aspergillus patch argues against a role for IL-17C in disease progression.

Calprotectin has been suggested to signal through TLR4 (33). Skin mRNA levels of S100A8 and A9 were significantly increased in mice repeatedly exposed to A. fumigatus, and TLR4 deficiency was associated with enhanced expression of S100A8 and A9. Indeed, S100A8/A9 skin levels correlate with skin barrier dysfunction, suggesting a role in disease pathogenesis. However, treatment with blocking Abs against S100A8 and A9 did not significantly alter TEWL in our model (data not shown).

In mice exposed to three patches of OVA over a 2-mo period, an increase in Th17 cytokines (IL-17A, IL-17F) was observed in the skin of OVA-patched BALB/c mice compared with control mice (34). The authors propose that the ability of epicutaneous OVA to induce a Th17 response is mediated by IL-23–expressing skin DC. In our model, no increase in IL-23p19 mRNA skin levels was observed following the third Aspergillus patch argues against a role for IL-23 in disease progression.

The protective role of MyD88 deficiency in our model supports a role for MyD88 signaling in disease exacerbation. Accordingly, proinflammatory cytokines and chemokine skin levels were markedly decreased. The impact on skin barrier was, however, more limited, suggesting that Aspergillus can induce skin disease independently of MyD88, possibly through the TRIF pathway.

TLR3 and TLR4, both of which signal through TRIF, have been implicated in wound healing: mice deficient in either TLR4 or TLR3 demonstrated impaired wound healing (41, 42). In human epithelial cells, wound repair was promoted by low doses of LPS, whereas high doses were deleterious (43). Similarly, when we exposed mice to high doses of LPS (1 μg/patch) in the presence of Aspergillus, disease was exacerbated (data not shown). Like many allergens, Aspergillus possesses considerable proteolytic activity. The proteolytic activity of cockroach and house dust mite allergens has been shown to delay skin barrier recovery following skin injury (44). One advantage of our study is that we used an allergen with no known interactions with TLR4. House dust mite–associated protease Derp2 can interact with TLR4 (45), whereas endotoxin contamination of OVA contributes to its ability to mount a Th2 response (46). Endotoxin levels within Aspergillus were very low (<0.1 ng/patch), but it remains possible that other TLR4 ligands may be present in the extract and may contribute to our phenotype.

In conclusion, in an experimental model of allergen-induced AD, defective TLR4 and TRIF signaling results in disease exacerbation, as evidenced by increased skin barrier dysfunction (TEWL) and epicutaneous sensitization associated with elevated proinflammatory cytokines, notably IL-1β and TNF-α.

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Disclosures
The authors have no financial conflicts of interest.

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