

Toll-like receptor 2 ligands promote chronic atopic dermatitis through IL-4-mediated suppression of IL-10

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Background: Atopic dermatitis (AD) is a T cell-mediated inflammatory skin disease, with T_H2 cells initiating acute flares. This inflamed skin is immediately colonized with *Staphylococcus aureus*, which provides potent Toll-like receptor (TLR) 2 ligands. However, the effect of TLR2 ligands on the development of T_H2-mediated AD inflammation remains unclear.

Objective: We investigated the progression of T_H2 cell-mediated dermatitis after TLR2 activation.

Methods: Using models for acute AD with T_H2 cells initiating cutaneous inflammation, we investigated the consequences of TLR2 activation. Dermatitis, as assessed by changes in ear skin thickness and histology, was analyzed in different BALB/c and C57BL/6 wild-type and knockout mouse strains, and immune profiling was carried out by using *in vitro* and *ex vivo* cytokine analyses.

Results: We show that T_H2 cell-mediated dermatitis is self-limiting and depends on IL-4. Activation of TLR2 converted the limited T_H2 dermatitis to chronic cutaneous

inflammation. We demonstrate that the concerted activation of TLR2 and IL-4 receptor on dendritic cells is sufficient for this conversion. As an underlying mechanism, we found that the combinatorial sensing of the innate TLR2 ligands and the adaptive T_H2 cytokine IL-4 suppressed anti-inflammatory IL-10 and consequently led to the exacerbation and persistence of dermatitis.

Conclusion: Our data demonstrate that innate TLR2 signals convert transient T_H2 cell-mediated dermatitis into persistent inflammation, as seen in chronic human AD, through IL-4-mediated suppression of IL-10. For the first time, these data show how initial AD lesions convert to chronic inflammation and provide another rationale for targeting IL-4 in patients with AD, a therapeutic approach that is currently under development. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: *Staphylococcus aureus, Toll-like receptor 2, innate immunity, IL-4, T_H2, atopic dermatitis, IL-10*

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Atopic dermatitis (AD) is a frequent inflammatory skin disease characterized by reduced skin barrier function, intracutaneous T-cell activation, itchy dermatitis, and susceptibility to cutaneous microbial and viral infections, and its prevalence has markedly increased during the past 3 decades. AD is thought to be based on (1) the genetic trait causing susceptibility and (2) environmental factors.¹⁻⁵ A detailed characterization of AD inflammation has revealed a biphasic cutaneous cytokine milieu with initial recruitment of IL-4-producing T_H2 cells, followed by a mixed phenotype in the chronic phase.^{6,7} Although cutaneous barrier dysfunction also contributes to T_H2 cell polarization, the T_H2 cell cytokine IL-4 further reduces the cutaneous barrier. Additionally, IL-4 suppresses antimicrobial peptide production and immune function, allowing cutaneous microbes to expand and persist.^{2,8-10} Next to this prominent communication between the epithelium and T cell-derived cytokines, professional antigen-presenting cells, especially activated dendritic cells (DCs), are also found in AD lesions and are thought to promote AD initiation and persistence by recruiting and activating T cells.¹¹

In contrast to the cascade of steps driving adaptive immunity during AD, much less is known about the role of innate immune activation. In the skin rapid activation of innate sentinels drives the first-line response to microbes. The key event in this process is the recognition of microbial pathogen-associated molecular patterns (PAMPs) by specific receptors, such as Toll-like receptors (TLRs), on sentinel cells.¹² After activation by innate immune signals, immune sentinels, such as DCs, orchestrate adaptive immune responses during infections, autoimmunity, allergy, and tolerance.^{13,14} To this end, pathogen recognition receptors on DCs recognize a broad spectrum of different

Abbreviations used

AD:	Atopic dermatitis
DC:	Dendritic cell
IL-4R:	IL-4 receptor
LTA:	Lipoteichoic acid
OVA:	Ovalbumin
Pam2:	Pam2Cys
PAMP:	Pathogen-associated molecular pattern
TLR:	Toll-like receptor
WT:	Wild-type

PAMPs.¹³ These innate immune sentinels detect more than 1 signal at a time, but only recently has it been appreciated that the combination of incoming signals is crucial for the outcome of immune responses.¹⁵ This is of special interest in the context of AD because AD skin lesions are heavily colonized with gram-positive *Staphylococcus aureus* and PAMPs from *S aureus* predominantly bind TLR2.^{2,16} Interestingly, levels of cutaneous TLR2 ligands strongly correlate with AD severity.¹⁷ Increased understanding of combinative innate immune sensing is required to comprehend the pathogenesis of chronic inflammatory diseases at interface organs, and studying such diseases can serve as a model to establish general mechanisms of innate inflammation.

In the present work we analyzed the consequences of innate immune sensing through TLR2 for T_H2 cell-mediated cutaneous inflammation. We used T_H2 cell-mediated dermatitis models because IL-4-producing T_H2 cells are found in early AD lesions when the skin encounters increasing innate TLR2 signals derived from *S aureus*. We found that a single exposure to TLR2 ligands converts T_H2-mediated transient dermatitis to chronic persistent cutaneous inflammation. These findings mimic the development of skin lesions in patients with AD. As the underlying mechanism, we demonstrated that the concerted activation of TLR2 and IL-4R on innate immune sentinels potently suppressed IL-10, thereby exacerbating T_H2-mediated dermatitis and initiating chronic inflammation. The most important immune skin sentinels are DCs, and we show that the concerted activation of TLR2 and IL-4R on DCs is sufficient to convert limited dermatitis into aggravated and persisting inflammation. Thus the combined sensing of innate immune signals together with the hallmark adaptive cytokine of early inflammation, IL-4, determined the outcome of this chronic immune response in patients with AD. For the first time, these data show how initial AD lesions are converted to chronic inflammation. In addition, these findings also provide another rationale for targeting IL-4 in patients with AD, a therapeutic approach that is currently under development.

METHODS**Mice**

BALB/c, C57BL/6, DO11.10, OT-II, and signal transducer and activator of transcription 6 (Stat6)-deficient mice were purchased from Charles River (Sulzfeld, Germany) or the Jackson Laboratory (Bar Harbor, Me). Tlr2^{-/-} BL/6 mice were provided by C. Kirschning (Duisburg, Germany) and backcrossed to the BALB/c background, MHCII^{-/-} mice were provided by L. Klein (Munich, Germany), and Il4ra^{-/-} mice were provided by A. Gessner (Erlangen, Germany). All mice were kept and bred under specific pathogen-free conditions in accordance with the guidelines of the Federation of European Laboratory Science Association. All animal experiments were in compliance with both European Union and German law and were approved by local authorities (Regierungspräsidium Tübingen, HT4/03, HT2/11, HT9/13).

Ovalbumin sensitization and adoptive T-cell transfer

Ovalbumin (OVA)-specific T cells were obtained from transgenic mice (DO11.10, OT-II) or from OVA-sensitized mice.¹⁸ CD4⁺ T cells were isolated with microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and expanded *in vitro*. For adoptive transfer, 1 × 10⁶ OVA-specific T_H2 cells with or without 5 μg of OVA protein (Hyglos, Regensburg, Germany) or 1.5 μg of OVA₃₂₃₋₃₃₉ peptide (EMC, Tübingen, Germany) were intracutaneously injected into the ear. *S aureus* lipoteichoic acid (LTA; 10 μg; obtained from T. Hartung, Konstanz, Germany) or Pam2Cys (Pam2; 2 or 4 μg; EMC) were included, where stated. DCs were exposed to 10 μg/mL Pam2 for 5 hours, washed twice with PBS, pulsed with 1 mg/mL OVA peptide for 1 hour, and washed twice with PBS. Controls were only pulsed with OVA. Where stated, DCs were incubated with 10 μg of IL-4/mL over night before Pam2 exposure. A total of 2 × 10⁵ DCs were applied intradermally together with 1 × 10⁶ T_H2 cells. For IL-10 supplementation in Il4ra knockout mice, 2 μg of IL-10 (PeproTech, Heidelberg, Germany) was added, and an additional 2 μg was injected 6 and 24 hours later. Control animals received PBS. For induction of endogenous OVA-specific T_H2 cells, DO11.10 mice were sensitized as previously described¹⁹ and challenged with OVA protein, followed by Pam2 injection. Ear thickness was measured with a micrometer (Kroeplin, Schlüchtern, Germany) and expressed as a change in ear thickness after treatment.

Cell culture

T-cell culture was performed, as previously described.²⁰ OVA-specific T_H2 cells were expanded by the addition of 1.5 ng/mL IL-4 (PromoCell, Heidelberg, Germany) and 10 μg of OVA or 2.5 μg/mL OVA₃₂₃₋₃₃₉ peptide. For T_H1 phenotyping, T cells were stimulated with 0.5 μg/mL phorbol 12-myristate 13-acetate (Sigma, Taufkirchen, Germany) and 1 μmol/L ionomycin (Sigma). Bone marrow-derived DCs were generated and cultured, as previously described,²¹ and stimulated with 10 μg of Pam2/mL or 10 μg of *S aureus* LTA/mL with or without 10 ng of IL-4/mL. Quantification of IL-4, IFN-γ, IL-10, IL-12p70 (BD PharMingen, Heidelberg, Germany), and IL-13 (eBioscience, San Diego, Calif) in the supernatant was performed by means of ELISA. For real-time analysis, DCs were cultured with the indicated additives with or without α-CD3/α-CD28-activated T_H2 cells in transwell plates for 6 hours.

Real-time quantitative PCR

Total RNA was extracted from *in vitro*-cultured DCs or from ears after adoptive transfer by using the RNA Kit (Machery & Nagel, Düren, Germany). RNA was reverse transcribed to cDNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Munich, Germany), according to the manufacturer's instruction. Quantitative real-time PCR was carried out with a LightCycler LC480 (Roche, Basel, Switzerland) by using SYBR Green Supermix (Roche). Data were presented normalized to the housekeeping gene β-actin and calculated as the difference from the value obtained after transfer of OVA alone, which was set as 1.

Statistical analysis

All data are presented as means ± SEMs and representative of at least 2 experiments. Statistical analysis was performed with the unpaired Student *t* test (2-tailed) or with 2-way repeated-measures ANOVA and the Bonferroni posttest. *P* values of less than .05 were considered statistically significant.

Additional information can be found in the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS**Self-limited allergen-induced dermatitis is mediated by IL-4**

Early immune reactions in AD skin are dominated by T_H2 cells and cytokines and are believed to predispose the AD skin for colonization by bacteria, such as *S aureus*. As a model for

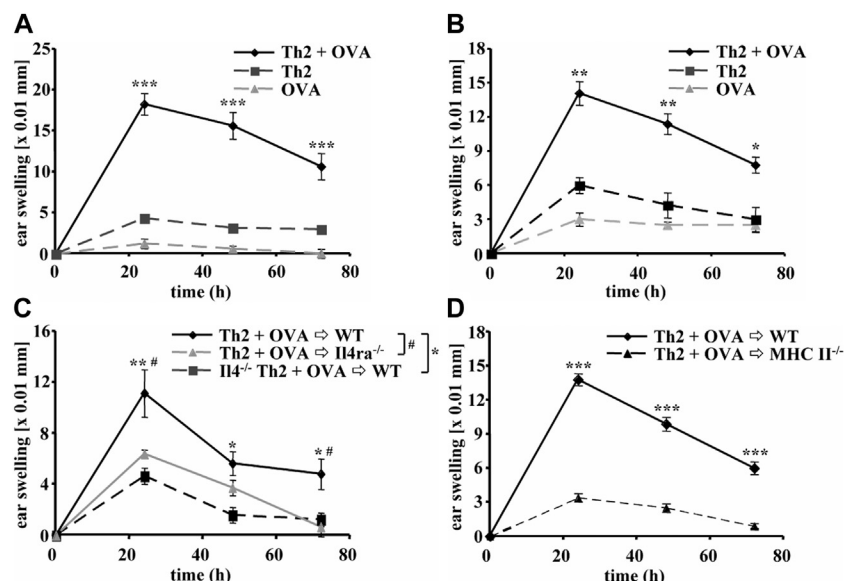


FIG 1. T_H2 -mediated dermatitis is self-limited and dependent on IL-4 and MHCII. **A** and **B**, Intracutaneous OVA-specific T_H2 cells mediate dermal inflammation in the ears of naive BALB/c (Fig 1, **A**) or C57BL/6 (Fig 1, **B**) mice quantified as changes in ear thickness. **C**, Transfer of WT or $IL4^{-/-}$ OVA-activated T_H2 cells in WT or $IL4^{-/-}$ mice (BALB/c background) demonstrated a strict dependence on IL-4. **D**, Complete abrogation of inflammation in $MHCII^{-/-}$ mice (C57BL/6 background). $n = 4$ -10. # $P < .05$, ** $P < .005$, and *** $P < .0005$.

the early phase of AD inflammation, OVA-specific IL-4-producing T_H2 cells were intracutaneously transferred with or without OVA into the ears of naive mice. Ear swelling was determined as the change in skin thickness to quantify dermatitis. Transfer of T_H2 cells with OVA provoked strong but self-limiting cutaneous inflammation, with a peak at 24 hours in both T_H2 -prone BALB/c (Fig 1, **A**) and C57BL/6 mice (Fig 1, **B**). OVA-specific T_H2 cells from wild-type (WT) or IL-4-deficient mice were generated to identify the underlying mechanisms. T_H2 polarization of $IL4^{-/-}$ T cells was determined based on IL-13 production, which was identical to cytokine secretion by WT T_H2 cells, whereas IL-4 production was lacking, as expected (see Fig E1 in this article's Online Repository at www.jacionline.org). Intracutaneous transfer of WT T_H2 cells and antigen resulted in a significant increase in ear thickness in WT mice that was dependent on T_H2 cell-derived IL-4 and host IL-4R. $IL4^{-/-}$ T_H2 cells did not induce dermatitis in WT mice, and similarly, WT T_H2 cells did not induce cutaneous inflammation in mice lacking the IL-4 receptor ($IL4ra^{-/-}$; Fig 1, **C**). Importantly, IL-4-mediated dermatitis was also strictly dependent on MHCII on recipient cells because it was completely abrogated in recipients deficient for MHCII (Fig 1, **D**). Thus our data demonstrate that antigen-activated T_H2 cells induce self-limited cutaneous inflammation, which is dependent on IL-4 signaling.

TLR2 ligands enhance and sustain T_H2 -mediated dermatitis through activation of skin-resident cells

S aureus is a dominant trigger of AD, providing high levels of different TLR2 ligands. One such ligand, LTA, was recently isolated from AD skin lesions and identified as a PAMP that correlates with AD severity.¹⁷ Therefore we analyzed the role of innate immune sensing of *S aureus*-derived PAMPs by exposing the skin to those TLR2 ligands. LTA significantly

enhanced dermatitis induced by OVA-specific T_H2 cells in BALB/c (Fig 2, **A**) and C57BL/6 (Fig 2, **B**) mice, indicating that innate immune sensing of *S aureus*-derived LTA contributes to AD inflammation. LTA was initially described as a ligand for TLR2,²² although it now appears that lipoproteins in LTA preparations mediate the TLR2-dependent effects of LTA.^{23,24} Therefore we next exposed the skin to the lipoprotein Pam2, another TLR2 ligand.²⁵ Like LTA, Pam2 significantly enhanced and sustained T_H2 -mediated dermatitis in BALB/c and in C57BL/6 mice, as shown by ear swelling (Fig 2, **C** and **D**, respectively). In addition, hematoxylin and eosin staining of skin sections at 48 hours demonstrated epidermal thickening (Fig 2, **E**, yellow arrow) and a massive dermal cellular infiltrate in these Pam2 conditions, exceeding findings in self-limited T_H2 dermatitis (Fig 2, **E**, white arrows). Interestingly, IFN- γ mRNA was upregulated in skin undergoing Pam2-exposed exacerbated inflammation, although not in self-limited T_H2 dermatitis skin (Fig 2, **F**). This upregulation could be due to endogenous T-cell bystander recruitment in addition to the transfer of T_H2 cells. Therefore we extended our analyses to a model in which endogenous T_H2 cells induced by OVA sensitization are recruited to the skin after antigen challenge.¹⁹ In this model, OVA-activated T_H2 cells led to short-lived dermatitis as well. In agreement with the results obtained with Pam2 in the transfer model, a single exposure to Pam2 in actively sensitized mice also induced severe and ongoing dermatitis, as assessed by measuring the increase in ear thickness (Fig 2, **G**) and by epidermal thickness and cellular infiltrates in histology (Fig 2, **H**). Next, we adopted our transfer model, in which T_H2 cells and OVA were administered first followed by exposure to Pam2 24 hours later because this might be closer to the situation in human AD. Exposure to Pam2 24 hours after T_H2 cell activation also significantly enhanced and prolonged

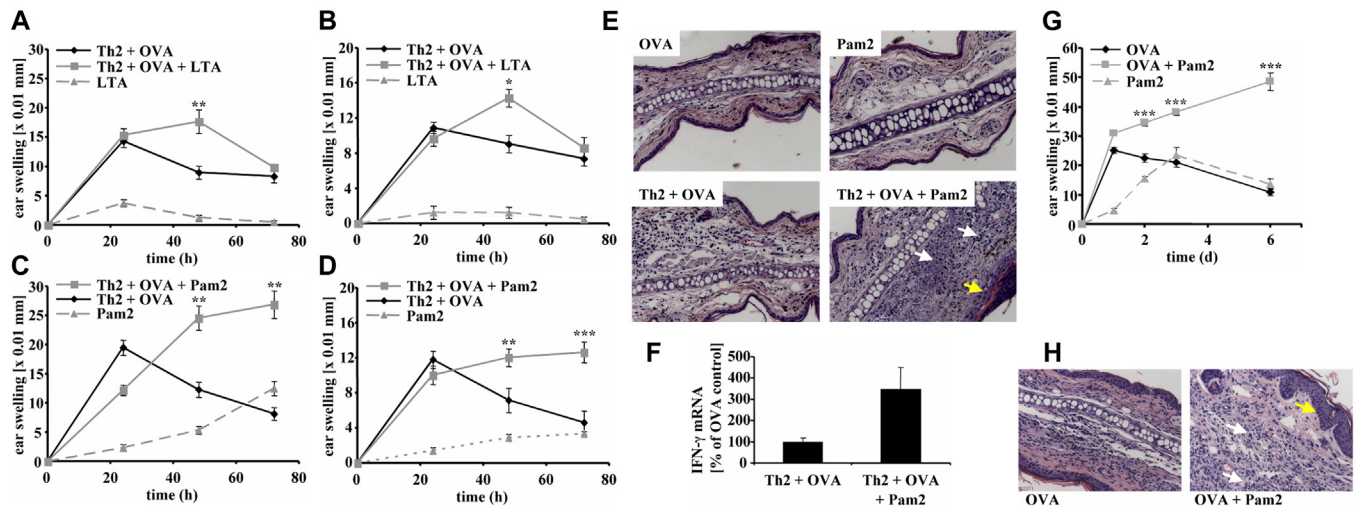


FIG 2. TLR2 ligands convert T_H2 -mediated acute dermatitis into exaggerated and persistent inflammation. **A-D**, Progression of T_H2 -induced dermatitis after exposure to TLR2 ligands in BALB/c (Fig 2, A and C) and C57BL/6 (Fig 2, B and D) mice is shown. LTA temporarily enhanced dermatitis (Fig 2, A and B). Pam2 converted dermatitis into persistent inflammation (Fig 2, C and D). **E**, Representative hematoxylin and eosin staining of histologic sections from C57BL/6 mice 48 hours after intracutaneous transfer. **F**, Relative IFN- γ mRNA level in ear skin 12 hours after transfer. **G** and **H**, OVA-sensitized DO11.10 mice (BALB/c background) challenged intracutaneously with OVA with or without Pam2. Cutaneous inflammation after OVA activation of endogenous T_H2 cells is shown in Fig 2, G, and histology (day 6) is shown in Fig 2, H. $n = 4-20$. * $P < .05$, ** $P < .005$, and *** $P < .0005$.

cutaneous inflammation, followed by significantly increased recruitment of $CD45^+$ leukocytes (see Fig E2 in this article's Online Repository at www.jacionline.org). Thus innate immune sensing through TLR2 enhanced and sustained skin inflammation caused by IL-4-producing T_H2 cells.

One possible target of TLR2 ligands are T cells because TLR2 can act as a costimulatory molecule for T-cell activation.²⁶ WT or $Tlr2^{-/-}$ T_H2 cells were adoptively transferred into either WT or $Tlr2^{-/-}$ mice, and skin inflammation was monitored thereafter to identify the dominant target cells for TLR2 ligands. In WT mice Pam2 enhanced and sustained dermatitis induced by OVA-activated T_H2 cells, irrespective of whether the T cells were TLR2 deficient (Fig 3, A). In contrast, TLR2 ligands completely failed to enhance and sustain T_H2 -induced dermatitis in $Tlr2^{-/-}$ mice, and the ear-swelling pattern in these mice was indistinguishable from the control transfer without TLR2 ligands (Fig 3, B). These data demonstrate that TLR2 ligands most likely target skin-resident antigen-presenting cells. Among these, the most important are DCs, which function as crucial sentinels of the immune system and link innate and adaptive immunity also in patients with AD.¹¹ Accordingly, we modified our protocol and transferred OVA-pulsed DCs with (Pam2-DC_{OVA}) or without (DC_{OVA}) Pam2 exposure together with OVA-specific T_H2 cells. Indeed, the transfer of OVA-pulsed DCs resulted in a limited antigen-induced dermatitis, whereas Pam2-DC_{OVA} caused significantly enhanced and prolonged inflammation (Fig 3, C). Aggravated skin inflammation was dependent on TLR2 activation of DCs because WT but not $Tlr2^{-/-}$ Pam2-DC_{OVA} exacerbated dermatitis in C57BL/6 mice (Fig 3, D) and BALB/c mice (see Fig E3 in this article's Online Repository at www.jacionline.org). Together, these data demonstrate that activation of DCs through TLR2 converts limited T_H2 -mediated dermatitis to an aggravated and persisting inflammation.

IL-4-induced suppression of IL-10 causes exacerbation and persistence of T_H2 -mediated dermatitis by TLR2 ligands

Given the critical role of IL-4 during early skin inflammation at the time of Pam2 encounter and of DCs for Pam2-induced dermatitis exacerbation, we next investigated the combined immune sensing of IL-4 and the TLR2 ligand Pam2 by DCs. To this end, DCs were activated with Pam2 with or without additional exposure to IL-4, as in AD skin. Cytokine levels were analyzed 24 hours later. The quality and functional consequences of immune responses are determined by the cumulative effect of differentially acting cytokines. Therefore we analyzed the main representatives of the proinflammatory and anti-inflammatory cytokines IL-12 and IL-10, respectively, to anticipate the immune consequences of such DCs. We found that Pam2-mediated activation of DCs induced IL-12 and IL-10. However, when mimicking the T_H2 -dominant milieu of early AD by adding IL-4, IL-12p70 levels were upregulated further in activated DCs, as expected.^{14,27} More importantly, IL-10 levels were significantly suppressed (Fig 4, A). This resulted in a proinflammatory shift of the cytokine milieu, as represented by the IL-10/IL-12 ratio (Fig 4, B). This suppression of IL-10 was indeed mediated by IL-4 because it was completely abolished in DCs with defective IL-4 signaling (see Fig E4 in this article's Online Repository at www.jacionline.org).

On the basis of these *in vitro* data, we asked whether this change of cytokine expression was also associated with the observed conversion of self-limited dermatitis into aggravated inflammation *in vivo* (Fig 2). To do this, we performed quantitative real-time PCR of ear skin 12 and 24 hours after the initiation of AD-like inflammation. As expected, Pam2 exposure upregulated IL-12 levels in the skin of recipient mice, with the highest levels detected 24 hours after challenge (Fig 5, A). Remarkably, and consistent with our *in vitro* results, cutaneous IL-10 expression

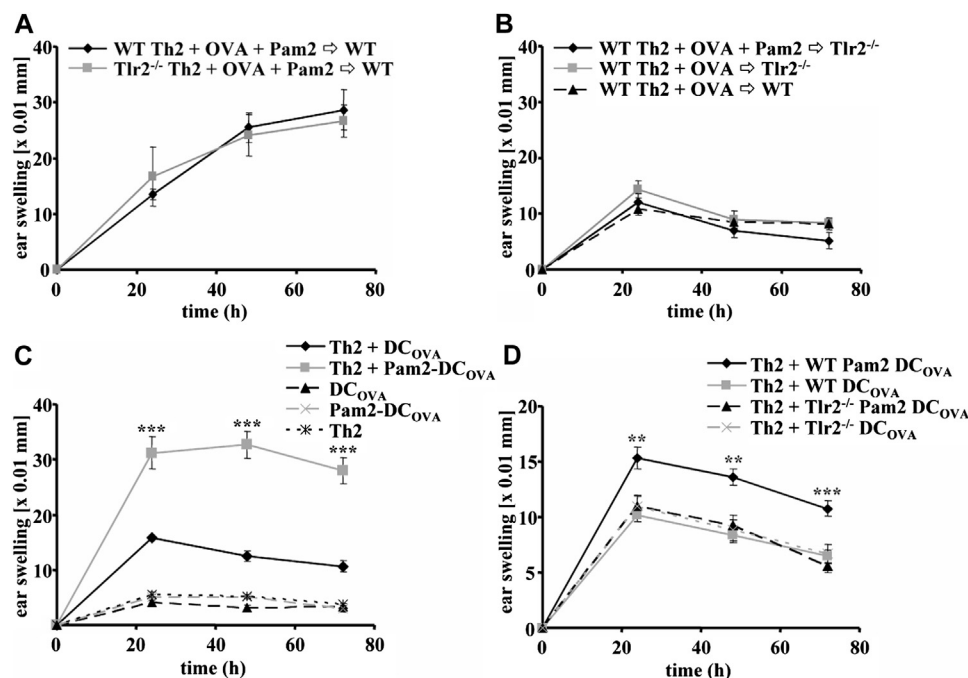


FIG 3. TLR2 activation of skin cells converts T_H2 -mediated acute dermatitis to enhanced and prolonged inflammation. **A** and **B**, Crossover experiment for persistent inflammation. Fig 3, **A**, shows Pam2-induced aggravated dermatitis in BALB/c mice after intracutaneous activation of either WT or $Tlr2^{-/-}$ T_H2 cells. Fig 3, **B**, shows self-limited acute dermatitis, as in OVA-treated control animals, despite exposure to Pam2 in $Tlr2^{-/-}$ recipients (done in parallel with Fig 3, **A**). **C**, Exacerbation and prolongation of dermatitis after transfer of T_H2 cells and Pam2-exposed OVA-pulsed DCs in C57BL/6 mice. **D**, Pam2-exposed WT but not $Tlr2^{-/-}$ DCs promote exacerbated inflammation in $Tlr2^{-/-}$ -C57BL/6 mice. $n = 4-10$. ** $P < .005$ and *** $P < .0005$.

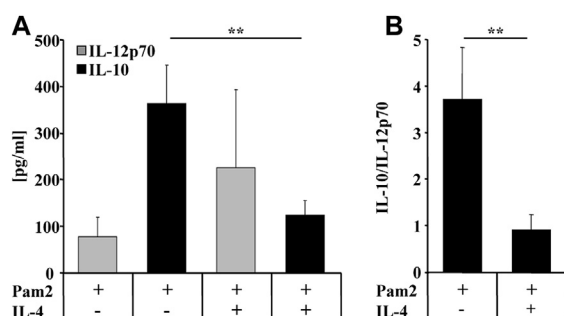


FIG 4. Suppression of TLR2 ligand-induced IL-10 by IL-4. **A**, DCs exposed to Pam2 produced intermediate levels of IL-12p70 and high levels of IL-10 (left). Coactivation of Pam2-exposed DCs with IL-4 significantly suppressed IL-10 secretion (Fig 4, **A**, right), resulting in a low IL-10/IL-12p70 ratio (**B**; average of 4 experiments). ** $P < .005$.

appeared earlier and was highest 12 hours after Pam2 exposure (Fig 5, **A**). This opposing regulation is best reflected by the cutaneous IL-10/IL-12 mRNA ratio: in mice with self-limited T_H2 -mediated dermatitis, IL-10 dominated skin inflammation at 24 hours (Fig 5, **B**, left). In contrast, the IL-10/IL-12 mRNA ratio was markedly reduced in dermatitis lesions that showed enhanced and sustained inflammation after TLR2 ligand exposure (Fig 5, **B**, right). Importantly, amplified IL-4 production, as detected by means of cutaneous IL-4 mRNA expression, was found in both self-limited T_H2 dermatitis and enhanced and sustained inflammation after Pam2 exposure (see Fig E5 in this article's Online Repository at www.jacionline.org). Therefore after innate

immune sensing of TLR2 ligands, T_H2 -derived IL-4 suppressed IL-10 levels, allowing TLR2 ligands to enhance and sustain AD inflammation. Indeed, IL-10 expression in Pam2-treated DCs was significantly reduced on coculture with IL-4-secreting T_H2 cells comparable with the suppression seen after direct addition of IL-4 (Fig 5, **C**). Intracutaneously transferred T_H2 cells and Pam2-DC_{OVA} were reconstituted with IL-10 to prove that IL-10 is indeed critical for the IL-4-mediated conversion of acute AD flares into persistent AD-like inflammation. Supplementation with IL-10 completely abrogated Pam2-induced dermatitis, which decreased to the level of inflammation without Pam2 activation (Fig 5, **D**). This demonstrated IL-10 to be a key regulator in the orchestration of adaptive T_H2 -mediated immune responses. Next, we carried out long-term analyses of Pam2-exposed dermatitis. Impressively, after a single exposure to Pam2, T_H2 -induced dermatitis increased over 5 days and remained significantly enhanced for more than 3 weeks (Fig 5, **E**). In summary, our data demonstrate that TLR2 ligands convert acute self-limited inflammation to chronic and persistent dermatitis through IL-4-mediated suppression of IL-10.

DISCUSSION

AD is based on a complex genetic trait, with skin barrier defects being among the most frequent functional abnormalities.^{3,28} The majority of patients with AD have increased IgE levels toward environmental antigens and T_H2 -biased T-cell immunity, which is based on both cutaneous barrier defects and an inherent immune bias toward T_H2 immunity.² A prerequisite for the

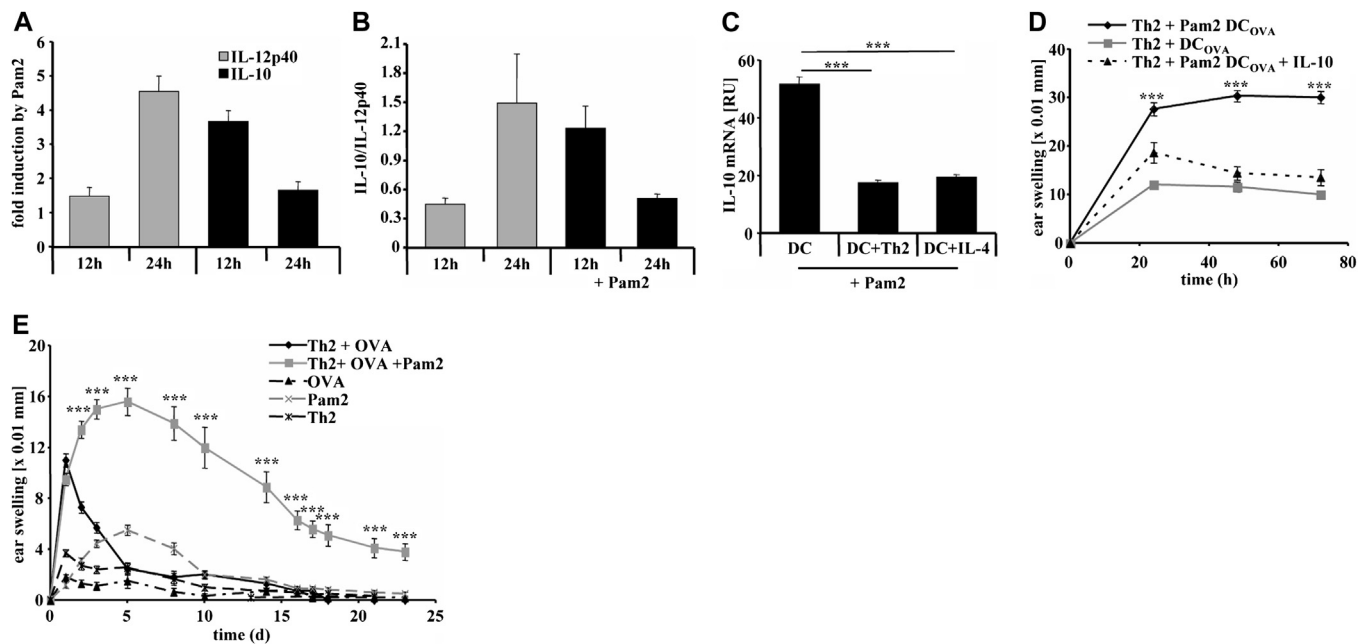


FIG 5. Suppression of IL-10 by coactivation of IL-4R and TLR2 converts acute dermatitis to chronic inflammation. **A** and **B**, Quantitative real-time PCR of cutaneous cytokines during conversion to persistent Pam2-induced dermatitis. Fig 5, **A**, shows Pam2-induced IL-12p40 and IL-10 expression. Fig 5, **B**, shows reduced IL-10/IL-12p40 ratio in persistent dermatitis. **C**, Suppression of IL-10 mRNA in Pam2-exposed DCs by means of coculture with activated T_H2 cells or the addition of IL-4. **D**, Reconstitution of IL-10 abrogated Pam2-induced aggravation of dermatitis after intracutaneous transfer of T_H2 cells, DCs, and OVA in *Il4ra*^{-/-} mice. **E**, A single Pam2 exposure converted T_H2-mediated dermatitis into exacerbated chronic inflammation in C57BL/6 mice. n = 6–10. ***P < .0005.

development of AD inflammation is the initial recruitment of IL-4–producing T_H2 cells to the skin on acute triggering factors, among them exposure to environmental allergens.^{29–31} However, the switch from acute AD flares to chronic cutaneous inflammation is not understood, and functional human *in vivo* studies are difficult to perform. Cutaneous colonization or infections with *S aureus* are found in almost all patients with AD, demonstrating a positive correlation between bacterial density and the severity of AD.³² Moreover, this correlation with AD severity is also valid for TLR2 ligands found on the skin.¹⁷ Therefore we hypothesized that TLR2 ligands play a major role in the conversion of acute AD inflammation to chronic dermatitis.

TLR2 has been associated with AD pathogenesis,² with the most recent work demonstrating that TLR2 contributes to skin barrier repair when acting on the epithelium.³³ To investigate how TLR2 ligands orchestrate cutaneous inflammation in patients with AD, we used a mouse model in which IL-4 is the dominant and functionally relevant cytokine, as in early AD. This allowed us to specifically focus on the effect of innate TLR2 signals in a T_H2-dominant adaptive immune response in the skin. Previous analyses concentrated on the role of TLR2 ligands for cutaneous T_H1 responses,³⁴ for which IFN- γ had been shown to mediate dermal thickening.³⁵ We showed that TLR2 ligands, as provided by *S aureus*, convert T_H2-mediated self-limited skin dermatitis into persistent and aggravated chronic inflammation. These analyses provide a rationale for the conversion of acute AD flares to chronic skin inflammation, which is observed in patients with AD. This conversion was driven by innate and adaptive signals that simultaneously activated immune sentinels of the skin. In general, TLR2 ligands are known to coinduce rather high levels

of IL-10.³⁶ This, under homeostatic conditions, might contribute to microbiota tolerance because gram-positive bacteria persist on the skin in the absence of inflammation. However, in the setting of IL-4–dominated inflammation, this upregulation is counterregulated by IL-4, which suppresses IL-10, leading to TLR2-mediated exacerbation of inflammation. Thus the combinative sensing of adaptive IL-4 together with innate TLR2 signals directly drives skin inflammation by suppressing IL-10. These newly identified consequences of coactivating signals shed light onto a hitherto neglected effect of combinatorial immune sensing.¹⁵ Moreover, the identification of IL-10 as a target cytokine of this immune orchestration confirms its dominant role as an important anti-inflammatory cytokine with immunomodulatory properties, limiting otherwise excessive immune responses.³⁷ It is well established that IL-10 regulates a variety of immune cells, including T_H2 cells.³⁸ Furthermore, IL-10 has even been used as a therapeutic agent for inflammatory diseases of the skin, such as psoriasis,³⁹ and the gut, such as Crohn disease.⁴⁰ Interestingly, an AD-like skin disease in NcNgA mice was effectively treated with IL-10,⁴¹ and IL-10 was also relevant for the amelioration of canine AD.⁴² These analyses support the concept that the adaptive cytokine IL-4 suppresses innately induced IL-10, which is responsible for the conversion of acute dermatitis to chronic and persistent inflammation.

These findings are highly relevant to patients with AD because *S aureus* is found on the skin of almost all such patients.³² In addition to *S aureus*, *Malassezia* species have been shown to be triggers of head-and-neck variants of AD, and herpes virus infections lead to severe AD complications.^{43–45} Importantly, in the context of our findings, innate signals from *Malassezia*

species and herpes viruses activate TLR2.^{46,47} This indicates that our findings on *S aureus*-derived TLR2 ligands might be of general importance and also functional in other settings in patients with AD.

Our findings also further emphasize that IL-4 is a promising target for AD therapy. It has long been known that IL-4 is the hallmark cytokine of T_H2 cells and early AD and that IL-4 downregulates antimicrobial peptides and cutaneous barrier function.^{6,7,48} We have now added a crucial new role for IL-4 in AD pathogenesis: IL-4 suppresses cutaneous IL-10 induced by innate signals and therefore promotes chronic AD. Until recently, however, IL-4 was targeted in patients with severe asthma⁴⁹ but not in those with AD. Now clinical trials report significant improvement of AD-related inflammation by subcutaneous application of a human mAb directed against IL-4Rα.⁵⁰⁻⁵³ Together with our data, this indicates that inhibiting IL-4R signaling also prevents IL-4-mediated suppression of IL-10, which is pivotal for persistent and chronic inflammation in patients with AD after the simultaneous activation of IL-4R and TLR2.

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Key messages

- T_H2 cell-mediated dermatitis is self-limiting and strictly depends on IL-4.
- TLR2 ligands convert self-limited T_H2-mediated dermatitis to chronic inflammation by activating skin-resident sentinels.
- TLR2 ligand-induced chronic dermatitis results from IL-4-mediated suppression of IL-10.

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METHODS

Skin cell analysis

Mouse ear tissue was incubated with Dispase II (Sigma-Aldrich) for 2 hours at 37°C. The dermis and epidermis were separated; digested for 30 minutes at 37°C in collagenase A (Serva, Heidelberg, Germany) or trypsin-

EDTA (Biochrom Berlin, Germany), respectively; and pooled again. Samples were given twice through a cell strainer to obtain single-cell suspension. After washing, cells were counted, stained for 30 minutes at 4°C, and analyzed by means of flow cytometry with an LSRII flow cytometer and FACSDiva Software (BD Biosciences, Heidelberg, Germany).

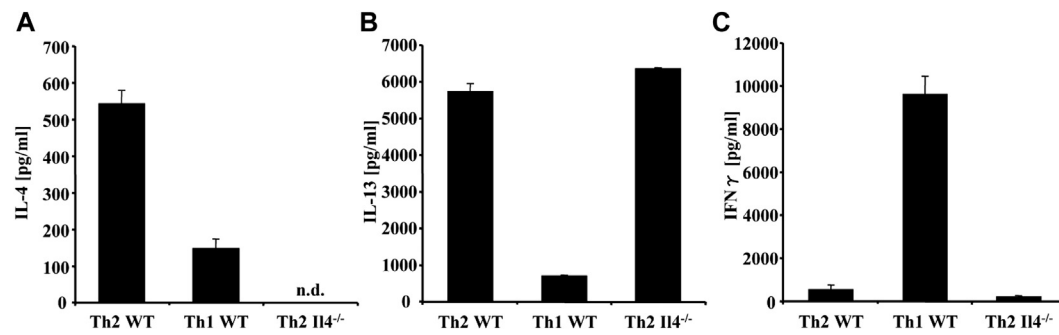


FIG E1. Phenotypic characterization of T_H2 cells. Characterization of WT and IL-4-deficient T_H2 cells used for intracutaneous transfer, as shown in Fig 2. Secretion of IL-4 (A), IL-13 (B), and IFN- γ (C) was determined by means of ELISA. For comparison, cytokine secretion by T_H1 cells is shown. *n.d.*, Not detectable.

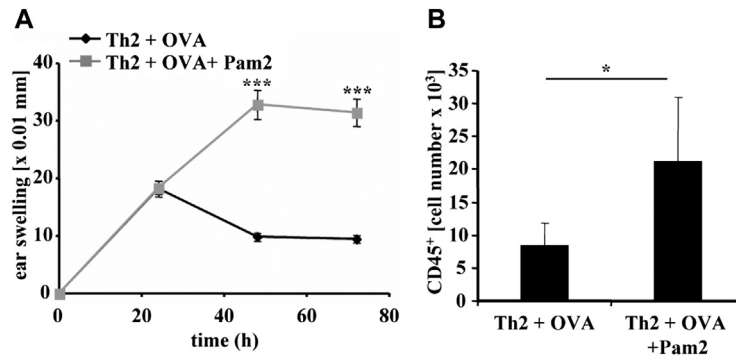


FIG E2. Pam2-induced exacerbated inflammation after T_H2 cell activation. **A**, T_H2 cells and OVA were transferred into the skin of BALB/c mice. Twenty-four hours later, some mice were additionally exposed to Pam2, which resulted in aggravated inflammation comparable with that seen after simultaneous application of T_H2 , OVA, and Pam2 (see Fig 2, C; $n = 8$). **B**, Ear skin analysis from the experiment depicted in Fig E2, A, showing increased numbers of $CD45^+$ cells in the Pam2-exposed condition. * $P < .05$ and *** $P < .0005$.

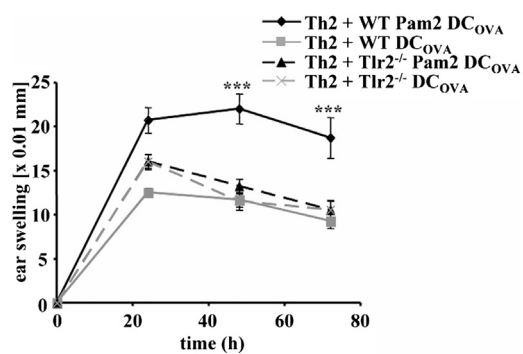


FIG E3. TLR2 activation of DCs converts T_H2-mediated acute dermatitis to enhanced and prolonged inflammation. Comparable with Fig 3, D, T_H2 cells and OVA-pulsed WT or Tlr2^{-/-} DCs with or without Pam2 exposure were transferred into Tlr2^{-/-} BALB/c mice, and ear swelling was measured thereafter. Pam2-exposed WT but not Tlr2^{-/-} DCs promoted exacerbated dermatitis (n = 10). ***P < .0005.

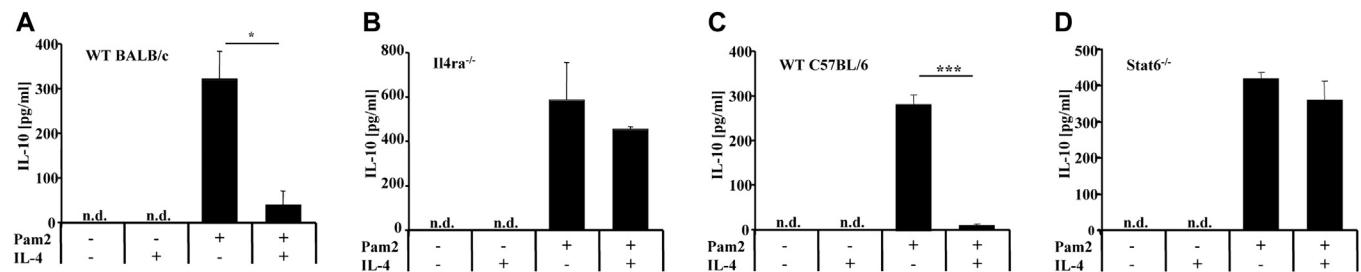


FIG E4. Suppression of IL-10 by IL-4 in Pam2-activated DCs. IL-10 secretion by bone marrow–derived DCs either from a BALB/c background (**A** and **B**) or a C57BL/6 background (**C** and **D**), as determined by means of ELISA. Addition of IL-4 significantly suppressed Pam2-induced IL-10 in WT DCs (Fig E4, **A** and **C**) but not in DCs with defective IL-4 signaling pathways (Fig E4, **B** and **D**). *n.d.*, Not detectable. * $P < .05$ and *** $P < .0005$.

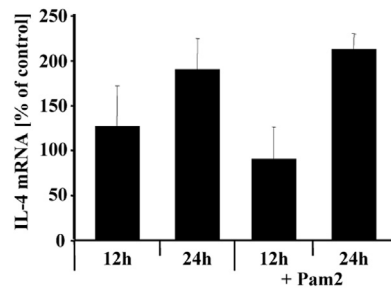


FIG E5. IL-4 expression in mouse ear skin. Relative IL-4 mRNA levels in mouse ear skin 12 and 24 hours after transfer of T_H2 cells and OVA with or without Pam2, corresponding to expression shown in Fig 5, A and B. Values were determined by using real-time PCR, normalized to β -actin, and shown relative to values obtained after transfer of OVA alone (n=6).