

ORIGINAL ARTICLE

Skin and Eye Diseases

Langerhans and inflammatory dendritic epidermal cells in atopic dermatitis are tolerized toward TLR2 activation

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Abstract

Background: The skin of atopic dermatitis (AD) patients presents a significant dysbalance of the microbiome with a high colonization by *Staphylococcus aureus* (*S. aureus*), which positively correlates with the severity of the disease.

Objective: Understanding the role of epidermal dendritic cells (DC) as link between the innate and the adaptive immune systems in AD.

Methods: Comparative phenotypic and functional analysis of TLR2 on Langerhans cells (LC) and inflammatory dendritic epidermal cells (IDEC) in organotypic models as well as freshly isolated cells from healthy and AD skin.

Results: *In situ* analysis of freshly isolated LC and IDEC from AD skin revealed decreased TLR2 expression compared to LC from healthy skin. In contrast to IDEC, LC from AD skin failed to display any evidence for *in situ* activation. Exposure to TLR2 ligand Pam3Cys resulted in maturation and increased migratory activity of LC from normal skin. LC and IDEC from AD were unresponsive to TLR2 ligand in that they failed to mature and displayed a high spontaneous migratory activity. Keratinocytes from both healthy and AD skin expressed similar levels of TLR2. The production of IL-6 and IL-10 was impaired by Pam3Cys in supernatants from AD skin. IL-18 was significantly higher in supernatants from AD skin and not influenced by TLR2 ligation, when compared to healthy skin.

Conclusion: Our results suggest that TLR2-mediated sensing of *S. aureus*-derived signals is strongly impaired in LC from AD skin. This phenomenon may partly contribute to the immune deviation in AD and the lack of *S. aureus* clearance.

KEYWORDS

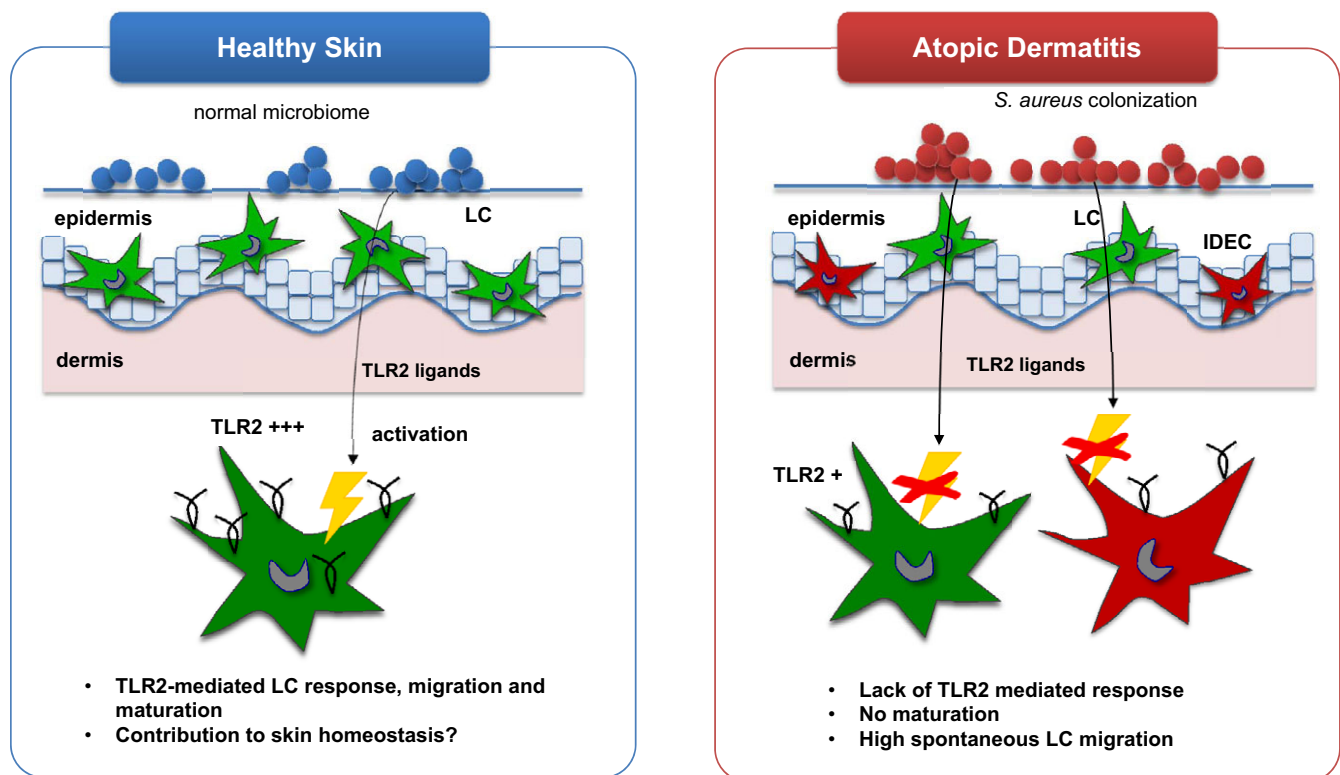
atopic dermatitis, inflammatory dendritic epidermal cells, Langerhans cells, microbiome, TLR2

1 | INTRODUCTION

The skin is continuously exposed to environmental factors including the skin microbiome. Healthy skin maintains its homeostasis by recognizing commensals and pathogens via receptors of the innate immune system, such as Toll-like receptors (TLR).^{1,2} Dendritic cells (DC)

represent the principal professional antigen-presenting cells and have been recognized to have a pivotal role in bridging innate and adaptive immunity.^{3,4} They express several TLR to sense danger signals from the external environment.⁵ TLR2 is a prominent member of the TLR family, and it is able to form homodimers or heterodimers with either TLR1 or TLR6. TLR2/1 recognizes triacylated, and TLR2/6 detects diacylated lipopeptides.⁶ TLR stimulation results in the activation and maturation of DC, characterized by expression of costimulatory

Kazumasa Iwamoto and Tim J. Nümm contributed equally to this work.



GRAPHICAL ABSTRACT

Langerhans cells (LC) and inflammatory dendritic epidermal cells (IDEC) in atopic dermatitis skin express less TLR2 compared to LC from healthy skin. Exposure to TLR2 ligand resulted in maturation and increased migratory activity of LC from normal skin. LC and IDEC in atopic dermatitis skin do not respond to TLR2 activation but displayed a high spontaneous migratory activity.

molecules and secretion of cytokines.^{4,7} The microenvironment can be modified by various skin cells, while the expression of IL-6 and IL-10 is induced by TLR2 ligation.⁸ IL-6 is a pivotal pro-inflammatory cytokine, which primes host defense against bacterial infection, while IL-10 has immunoregulatory functions that are important in protecting the host from infection-associated immunopathology.⁹⁻¹² IL-18 is associated with disease severity in atopic dermatitis (AD), it can be produced by epidermal cells, and it is known to induce cell migration.¹³⁻¹⁵ DC can activate and polarize T cells depending on the tissue microenvironment to which the DC might contribute and are exposed to.^{16,17} Langerhans cells (LC) are the main DC in normal epidermis while AD is additionally colonized by inflammatory dendritic epidermal cells (IDEC).¹⁸

AD patients show a dysbalance of the skin microbiome and have significantly more frequent *Staphylococcus aureus*-induced bacterial infections.^{19,20} It is assumed that TLR2 plays a crucial role in the recognition and defense toward *S. aureus*.²¹ Cutaneous TLR2 activation was reported to suppress T-cell immune responses.^{22,23} In impetiginized skin from patients with AD, a biologically active amount of TLR2 ligand lipoteichoic acid was detected.²⁴ However, the TLR2 expression and function on DC from human skin remain elusive.^{25,26} We hypothesized that LC may contribute to the lack of controlling *S. aureus* colonization by an alteration of their TLR2-mediated sensing.

2 | METHODS

2.1 | Healthy donors and AD patients

Normal skin was obtained from healthy, nonatopic individuals ($n = 57$; mean age $\pm$ SD, 46 ± 14.5 years) undergoing plastic surgery at the University of Bonn. Patients with moderate-to-severe AD (SCORAD > 30 ; $n = 24$; mean age $\pm$ SD, 41.3 ± 14.9 years; mean total IgE $\pm$ SD, $8,708 \pm 9,522$ IU/mL) were diagnosed according to the criteria of Hanifin and Rajka.²⁷ The total serum IgE levels were measured by the core facility of the University Hospital Bonn using the ImmunoCAP system. This study was approved by the local ethics committee of the University of Bonn and performed in accordance with the declaration of the Helsinki principle. Informed consent was obtained from all of the participants in this study.

2.2 | Reagents

The medium used for the culture of skin specimens was very low endotoxin—RPMI1640 (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich, St. Louis, MO, USA) and 1% antibiotics/antimycotics (Gibco, Karlsruhe, Germany). TLR1/2 ligand N-palmitoyl-S-[2, 3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-

cysteiny]-[S]-seryl]-[S]-(lysyl)3-[S]-lysine (Pam3Cys) was obtained from EMC microcollections (Tübingen, Germany). Details of the antibodies used are given in the Table S1. 7-amino-actinomycin D (7-AAD) was from Sigma-Aldrich (St. Louis, MO, USA).

2.3 | Preparation of *ex vivo* skin and epidermal cell suspensions

After obtaining healthy skin, split-thickness samples (0.4 mm) were immediately prepared using a dermatome. Biopsies of lesional AD skin were prepared by shave biopsy (average thickness of 0.4 mm) after local anesthesia. To increase the quality and reproducibility of the experimental setup, the investigated skin samples were adjusted to a standardized area with a 6-mm punch biopsy (Figure S1). Thus, migration experiments and cytokine analysis were related to the same sample size. For flow cytometric analysis, Epidermal cell (EC) suspensions were prepared by trypsinization as described previously.²⁸

2.4 | Culture and stimulation of *ex vivo* skin

Ex vivo skin specimen (one 6-mm biopsy/well) were cultured (Figure S2) in 1 mL medium in 24-well plates for 24 h at 37°C. After culture, the *ex vivo* skin samples were subjected to 0.5% trypsin for 1 h at 37°C, to obtain EC suspensions for flow cytometry analysis as described previously.²⁸ For stimulation of *ex vivo* skin, a Pam3Cys concentration of 10 µg/mL was used. This concentration was found to be optimal in preliminary experiments (data not shown).

2.5 | Migration experiment

Ex vivo skin was cultured in the presence or absence of Pam3Cys. After 24 h and 48 h, the number of migrated cells in the culture

medium was counted after excluding dead cells by trypan blue staining. Migrated cells were stained against surface CD1a, and intracellular Langerin to discriminate migrated LC from other migrated cells such as IDEC and CD1a^{pos} dermal DC. The percentage of migrated LC of all migrated cells was analyzed by flow cytometry. Finally, the number of migrated LC was calculated as follows: total number of migrated cells × percentage of migrated LC.

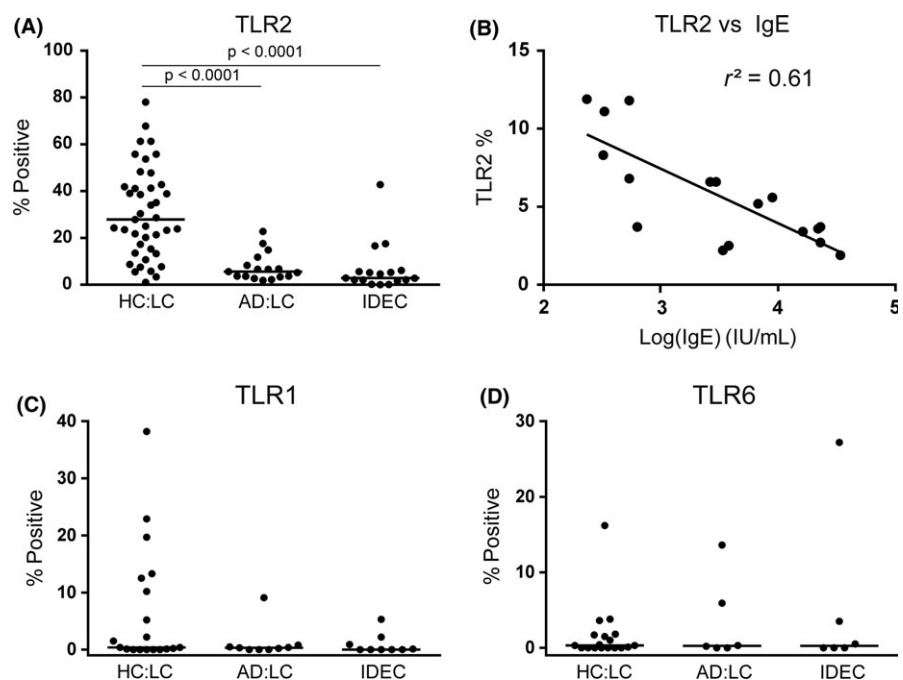
2.6 | Flow cytometric analysis

Cells from EC suspensions were stained as described elsewhere.²⁸ Antibodies used are described in the Table S1. LC and IDEC were gated from the EC suspensions by the expression of both CD1a^{pos} and Langerin^{pos}, and Langerin^{neg}, respectively, after dead cell exclusion by 7-AAD (Figure S3). For intracellular staining, cells were permeabilized using the BD Cytofix/Cytoperm kit (Becton Dickinson, San Jose, CA, USA). Surface and intracellular markers were measured using a FACS Canto and analyzed with the FACS Diva software (Becton Dickinson, San Jose, CA, USA) and FlowJo 10.4.1 (TreeStar, Ashland, OR, USA). The results are given in percentage of positive cells. For some markers (CD83, CD86, and MHC class II), the relative fluorescence index (rFI) was calculated as follows: $(\text{mean fluorescence intensity (MFI)}_{\text{surface marker}} - \text{MFI}_{\text{isotype control}}) / \text{MFI}_{\text{isotype control}}$.

2.7 | ELISA

The culture supernatants from *ex vivo* skin cultures were collected after 24 h and stored at −80°C. Cytokines were analyzed by ELISA kits for IL-6, IL-10 (Invitrogen, Camarillo, CA, USA) and IL-18 by LEGENDplex™ (Biolegend, San Diego, CA, USA).

FIGURE 1 Freshly isolated epidermal dendritic cells from atopic dermatitis (AD) skin express less Toll-like receptors 2 (TLR2) *in situ* than those from healthy skin. Epidermal cell suspension was prepared from AD and healthy skin and stained with antibodies against CD1a and Langerin to discriminate between inflammatory dendritic epidermal cells (IDEC) and Langerhans cells (LC) in flow cytometry analysis. Expressions of A, TLR2 (healthy controls [HC], n = 41; AD, n = 17); C, TLR1 (HC, n = 19; AD, n = 9), and D, TLR6 (HC, n = 18; AD, n = 6) are shown. The Mann-Whitney *U* test was used for statistical analysis. B, The linear regression of surface TLR2 expression from AD LC against total serum IgE is shown.



2.8 | Statistical analyses

Statistical analyses were performed by SPSS ver.24 (IBM, Armonk, NY, USA). Some of the results were presented as a box plot. The box covers the samples from the first to the third quartiles, the thick line indicates the median value, and the cross represents the mean. The whiskers outside of the box extend to the highest and lowest value within 1.5 times the height of the box. Outliers are indicated as filled circles (●). Significances for paired samples were calculated by the Wilcoxon rank sum test, and for independent samples, we used the Mann-Whitney *U* test.

3 | RESULTS

3.1 | Lower expression of TLR2 on Langerhans cells and inflammatory dendritic epidermal cells in AD skin

In a first approach, we examined the TLR2 expression on freshly isolated epidermal DC from healthy and AD skin. While freshly isolated LC from normal skin expressed significant amounts of TLR2, a markedly reduced expression of TLR2 was observed on LC freshly isolated from AD skin (Figure 1A). IDEC showed a similarly low TLR2 expression as LC from AD (Figure 1A). The total serum IgE level of AD patients was inversely correlating with TLR2 expression (Figure 1B). There was no significant difference in the expression of neither TLR1 nor TLR6 which were expressed similarly on LC from HC

and AD as well as on IDEC (Figure 1C and D, see corresponding histogram in the Figure S4). The expression of TLR1 and TLR6 was hardly detectable, while the TLR2 expression on keratinocytes did not differ in healthy and AD skin *in situ* (data not shown).

3.2 | High spontaneous migratory activity of Langerhans cells from AD

We then explored the migratory behavior of LC using an *ex vivo* skin culture model in the absence or presence of Pam3Cys mimicking *S. aureus*. While spontaneous migration of LC from healthy skin was hardly detectable, the addition of Pam3Cys leads to a significant induction of LC migration after 48 h (Figure 2). In contrast, LC from untreated AD patients showed a strong spontaneous migratory activity. After 24 h, the spontaneous migration rate of AD skin was significantly higher than that of LC from Pam3Cys-stimulated healthy skin. This effect was even further increased after 48 h. There was no evidence for further increase in the migration rate of LC from AD skin in response to Pam3Cys (Figure 2). Compared to LC, a similar migratory activity of Langerin^{neg} cells, including dermal DC and IDEC (only in AD skin), was observed (Figure S5).

3.3 | Evidence for *in situ* activation of inflammatory dendritic epidermal cells but not Langerhans cells

As LC from AD skin showed a high migration rate and a low TLR2 expression, we analyzed freshly isolated epidermal cells to verify a potential pre-activation by *S. aureus in situ*. Interestingly, freshly isolated LC from AD and normal skin expressed similar CD83 and MHC class II levels, suggesting that LC were not pre-activated *in situ* (Figure 3A and B). In contrast, freshly isolated IDEC expressed a phenotype compatible with matured DC, that is, higher expression of CD83, MHC class I, and MHC class II (Figure 3A and B). As an internal control of the IDEC population, we analyzed CD36 and CD206 expression (data not shown). Both were highly expressed on IDEC as described previously.²⁹ The corresponding histogram overlays from representative donors are depicted in the Figure S6.

3.4 | Langerhans and inflammatory dendritic epidermal cells in AD *ex vivo* skin lack phenotypic maturation responses to TLR2 ligation

Next, we studied the behavior of LC upon TLR2 ligation. In healthy *ex vivo* skin the full maturation status of LC was acquired upon stimulation with Pam3Cys, witnessed by increased CD83 and MHC class II (Figure 4B and C) as well as CD80 and CD86 (Figure S7). Despite the strong spontaneous migration rate of AD skin, LC and IDEC did not show any sign of an induced maturation status upon exposure to Pam3Cys as witnessed by lack of alteration of CD83 and MHC class II expression (Figure 4B and C) as well as CD80 and CD86 (Figure S4). Furthermore, Pam3Cys reduced the TLR2 expression on LC of healthy skin but not on LC and IDEC of AD skin

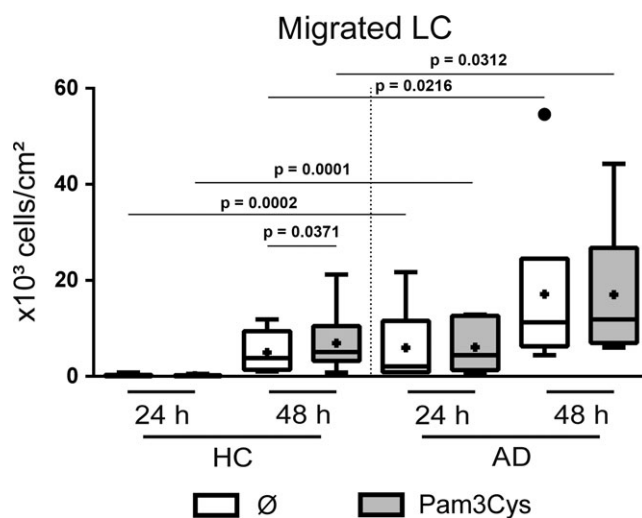


FIGURE 2 Toll-like receptors 2 (TLR2) ligation enhances the emigration of Langerhans cells (LC) from healthy, but not from atopic dermatitis (AD) skin. *Ex vivo* human skin was cultured without (open bars) or with Pam3Cys (gray bars) for 24 h and 48 h, respectively. Emigrated cells were counted after the indicated time points, and dead cells were excluded by trypan blue staining. The number of migrated LC was calculated based on flow cytometry analysis of CD1a and Langerin expression of healthy controls [HC] (left panel, *n* = 10) and AD (right panel, *n* = 6). The Wilcoxon matched-pairs signed-rank test was used for connected and the Mann-Whitney *U* test independent samples.

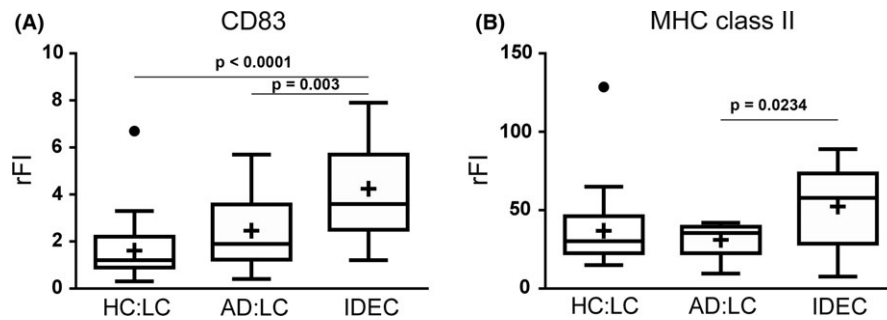


FIGURE 3 Inflammatory dendritic epidermal cells (IDEC) but not Langerhans cells (LC) are activated *in situ*. Epidermal cell suspension was prepared from atopic dermatitis (AD) and healthy skin and stained with antibodies against CD1a and Langerin to discriminate between IDEC and LC in flow cytometry analysis. Cell surface expression of A, CD83 (healthy controls [HC], $n = 39$; AD, $n = 16$) and B, MHC class II (HC, $n = 27$; AD, $n = 8$) was analyzed. The Wilcoxon matched-pairs signed-rank test was used for connected and the Mann-Whitney U test independent samples.

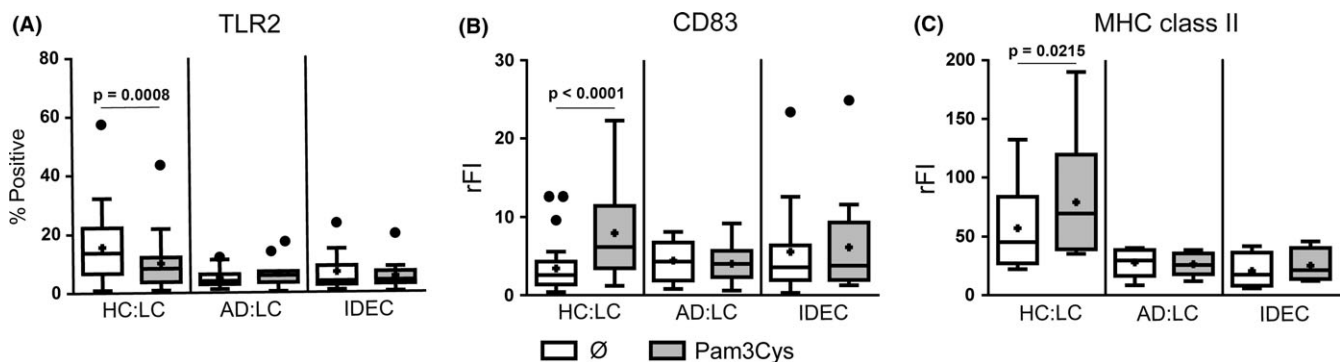


FIGURE 4 Toll-like receptors 2 (TLR2) ligation enhances the maturation of Langerhans cells (LC) in healthy but not in atopic dermatitis (AD) skin. *Ex vivo* human skin was cultured without (open bars) or with Pam3Cys (gray bars) for 24 h. Epidermal cell suspensions were generated and cell surface expression of A, TLR2 (healthy controls [HC], $n = 21$; AD, $n = 13$), B, CD83 (HC, $n = 25$; AD, $n = 13$) and C, MHC class II (HC, $n = 13$; AD, $n = 5$) on LC and inflammatory dendritic epidermal cells (IDEC) was analyzed by flow cytometry. The Wilcoxon matched-pairs signed-rank test was used for statistical analysis.

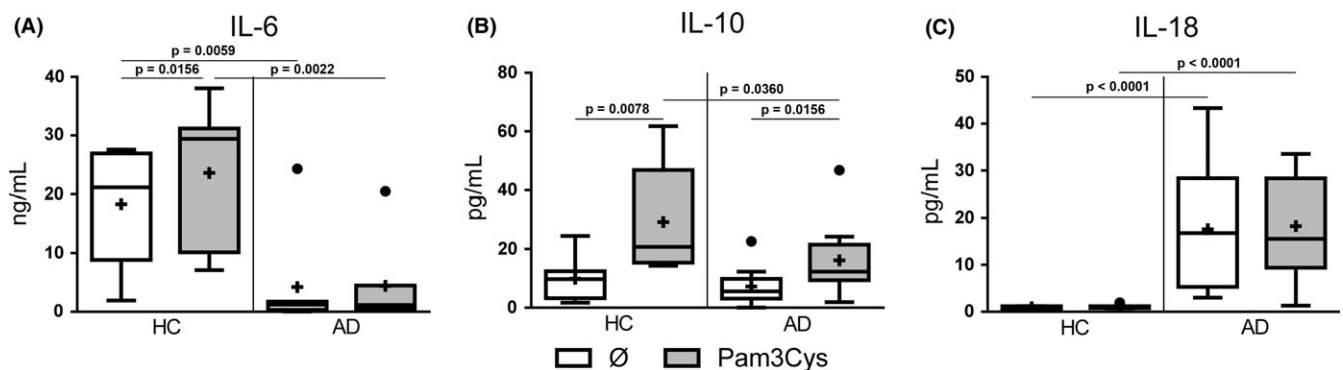


FIGURE 5 Impaired IL-6 and IL-10 production of atopic dermatitis (AD) *ex vivo* skin after Toll-like receptors 2 (TLR2) ligation. *Ex vivo* human skin was cultured without (open bars) or with Pam3Cys (gray bars) for 24 h. Cytokine expression of A, IL-6 (healthy controls [HC], $n = 8$; AD, $n = 7$), B, IL-10 (HC, $n = 8$; AD, $n = 9$) and C, IL-18 (HC, $n = 7$; AD, $n = 14$) was analyzed in the supernatant by ELISA. The Wilcoxon matched-pairs signed-rank test was used for connected and the Mann-Whitney U test independent samples.

(Figure 4A). The corresponding histogram overlays from representative donors are depicted in the Figure S8-S10. In order to examine whether the lack of TLR2-induced maturation in AD is due to the lower expression of TLR2, we additionally analyzed LC from HC and

AD donors with similar expression of the receptor. Only in HC CD83 was increased after Pam3Cys treatment (Figure S11), suggesting a tolerance to TLR2 activation rather than a result of decreased TLR2 expression in AD.

3.5 | Differential response of AD *ex vivo* skin to TLR2 ligation

Various cells in the dermis and epidermis including keratinocytes and fibroblasts dictate the skin microbiome and thereby have an impact on DC.³⁰ To evaluate an overall response of the skin upon stimulation by *S. aureus*-derived signals, we applied the TLR2 ligand Pam3Cys on *ex vivo* skin samples and measured the cytokines in the supernatants. The TLR2 expression on keratinocytes did not change with Pam3Cys treatment (data not shown). The amount of IL-6 was significantly enhanced by TLR2 ligation in healthy but not in AD skin, whereas AD skin produced 20 times less IL-6 than the base level of healthy skin and was not further increased upon TLR2 ligation (Figure 5A). In addition, the production of IL-10 in the supernatant was significantly enhanced by TLR2 ligation in healthy and AD skin, while AD skin expressed IL-10 at much lower levels (Figure 5B). However, the constitutive secretion of IL-10 was strongly reduced in AD when compared to healthy skin. IL-18 was considerably higher expressed in the supernatant of AD compared to healthy skin, while TLR2 ligation did not alter the IL-18 expression significantly in both groups (Figure 5C).

4 | DISCUSSION

In this study, we demonstrate that, in contrast to keratinocytes, freshly isolated epidermal LC and IDEC from AD skin displayed lower TLR2 expression, while only IDEC were highly matured in "steady state." The Pam3Cys treatment of *ex vivo* skin mimicking *S. aureus*-derived signals revealed that LC and IDEC of AD skin did tolerate the TLR2 ligation in terms of maturation and migration, while cytokine production in the supernatant from *ex vivo* skin was impaired.

AD patients suffer from an imbalance of the skin microbiome, which may modulate TLR2 expression and signaling due to desensitization by prolonged exposure to its ligands.³¹ *Staphylococcus epidermidis* is the predominant *Staphylococcus* species in healthy skin and is able to inhibit *S. aureus* colonization by a lipopeptide, which activates TLR2/6 and CD36, induces MAPK signaling and increases antibacterial defense against bacterial infection.^{32,33} In contrast, *S. aureus* seems to activate the TLR1/2 pathway leading to NF- κ B activation and is able to secrete the virulence factor PSM α which increases the inflammation.^{33,34} During an AD flare the skin microbiome is changing, increasing colonization of *S. aureus* and *S. epidermidis*, while *S. epidermidis* is not able to control *S. aureus* anymore.³⁵ We show that freshly isolated LC and IDEC of AD skin displayed a strong reduction in *in situ* TLR2 expression compared to healthy individuals, as reported in macrophages.³⁶ On the other hand, others have shown an increased TLR2 expression on AD monocytes.³⁷ Furthermore, it has been reported that the TLR2 promoter polymorphism A-16934T has no impact on TLR2 mRNA expression on monocytes.³⁸ It has been shown earlier that IL-4 and IL-13 reduce the TLR2 expression on human monocytes and mice DC, since Th2 cytokines are dominant in AD.³⁹⁻⁴² However, it has

been reported that IL-4 and IL-13 do not influence TLR2 mRNA, while IFN- γ is able to upregulate TLR2 on human intestinal epithelial cells.⁴³ This indicates that the Th2 microenvironment is able to influence the base level of TLR2, which may contribute to the low expression in AD LC in our data. Interestingly, a TLR2-deficient AD mouse model does not influence Th2 polarization, while the Th1 cytokine IFN- γ is remarkably reduced when compared to wild-type mice.⁴⁴ As a consequence, these mice were not able to polarize naïve T cells to Th1.

In our study, Pam3Cys was able to induce the TLR2 dependent maturation of LC in healthy skin, while AD LC and IDEC lacked maturation witnessed by constant levels of MHC class II, CD83, CD80, and CD86 despite TLR2 ligation. The low TLR2 expression on AD LC may not per se be the cause of the failed activation, as the low TLR2-expressing LC from healthy skin are able to respond to Pam3Cys (Figure S11). Furthermore, *in vitro* experiments with LC generated from CD34 stem cells express similar low TLR2 levels compared to AD LC and treatment with Pam3Cys results in strong activation thereof.⁷ It has been reported that cultivated human primary keratinocytes from hair follicles of healthy and AD patients express similar levels of TLR2 but the outcome of pro-inflammatory mediators by Pam3Cys treatment is altered, which suggests an impaired signaling in AD patients.⁴⁵ Furthermore, the TLR2 polymorphism R753Q is associated with a severe phenotype and higher levels of IgE in AD patients.⁴⁶ Others showed an altered function of MyD88 signaling in mice macrophages bearing the TLR2 polymorphism R753Q.⁴⁷ It has been reported that the frequency of R753Q is higher in AD children, while others showed a similar distribution in healthy children and concluded that the association between AD and R753Q is lacking.^{48,49} Nevertheless, it is unlikely that the polymorphisms are playing a major role in our data, as the prevalence of the R753Q polymorphism is around 10%.⁵⁰

The TLR2-dependent migration of LC out of healthy skin is most likely due to induced maturation by Pam3Cys as discussed by others.⁵¹ However, the missing activation of LC by Pam3Cys fails to explain the strong spontaneous migration in AD (Figure 2). It is more likely caused by an elevated base level of IL-18, which was independent from TLR2 ligation in our experimental setup (Figure 5C). IL-18 is strongly expressed in the serum of AD patients and it is known to induce LC migration.^{14,52} IL-18 can be released by keratinocytes and IDEC-like DC and has a pleiotropic effect that stimulates both Th2 and Th1 responses, depending on the microenvironment.⁵³⁻⁵⁵ This may induce a cycle of events, where IL-18 triggers Th2 cells, which in turn modulate TLR expressions by their locally produced cytokines, as discussed above. Additionally, the C-C chemokine receptor type 6, which regulates migration activity, is reduced on AD LC compared to HC LC.⁵⁶ This suggests that they might migrate more easily.

Various skin cells including dermal DC, LC, keratinocytes and fibroblasts shape the skin microenvironment and are able to activate immune cells.^{30,57} We showed that healthy *ex vivo* skin was able to increase levels of IL-6 and IL-10 after TLR2 ligation. In contrast, AD *ex vivo* skin produced much lower levels of IL-10 and IL-6, whereas

IL-6 was not altered by Pam3Cys. Human primary keratinocytes and macrophages from AD patients show lower levels of IL-6 after Pam3Cys challenge compared to HC.^{36,45} The lack of response we observed herein may be due to repeated TLR engagement *in situ*, as *S. aureus* may constantly trigger the immune cells in AD skin. This hypothesis is indeed confirmed by *ex vivo* studies of human monocytic cell lines and monocyte-derived DC, where the cells produce less cytokines upon TLR2 stimulation when exposed repeatedly to TLR2 ligands.^{58,59} Intrinsic alterations in TLR2 response have been suspected previously in monocytes, macrophages, and keratinocytes from AD patients.^{36,45,60,61} Taken together, our data support that desensitization, tolerance or intrinsic alterations of epidermal LC to TLR2 response, is key in AD.

Our findings further support the hypothesis that patients with AD are more susceptible to cutaneous pathogens due to defects in TLR2 expression and lack of downstream signaling in epidermal DC. This may also have a substantial impact on the overall T2 type of immune response in AD and the sensitization through the epidermal compartment. Further investigation of the interplay between TLR2 and *S. aureus* in inflamed skin may open up new therapeutic approaches.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

K.I. and T.N. designed and performed the experiments with skin samples. N.H. and N.L. designed and performed cytokine experiments. K.I., T.N., and S.K. wrote the manuscript. T.B. coordinated the project, prepared skin samples from patients with AD, supervised all experiments, and edited the manuscript.

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REFERENCES

- Nestle FO, Di Meglio P, Qin JZ, Nickoloff BJ. Skin immune sentinels in health and disease. *Nat Rev Immunol*. 2009;9:679-691.
- Castan L, Magnan A, Bouchaud G. Chemokine receptors in allergic diseases. *Allergy*. 2017;72:682-690.
- Wollenberg A, Wen S, Bieber T. Phenotyping of epidermal dendritic cells: clinical applications of a flow cytometric micromethod. *Cytometry*. 1999;37:147-155.
- Novak N, Koch S, Allam JP, Bieber T. Dendritic cells: bridging innate and adaptive immunity in atopic dermatitis. *J Allergy Clin Immunol*. 2010;125:50-59.
- Gros E, Novak N. Cutaneous dendritic cells in allergic inflammation. *Clin Exp Allergy*. 2012;42:1161-1175.
- Buwitt-Beckmann U, Heine H, Wiesmuller KH, et al. TLR1- and TLR6-independent recognition of bacterial lipopeptides. *J Biol Chem*. 2006;281:9049-9057.
- Herrmann N, Koch S, Leib N, et al. TLR2 down-regulates FcεpsilonRI and its transcription factor PU.1 in human Langerhans cells. *Allergy*. 2013;68:621-628.
- Jang S, Uematsu S, Akira S, Salgame P. IL-6 and IL-10 induction from dendritic cells in response to Mycobacterium tuberculosis is predominantly dependent on TLR2-mediated recognition. *J Immunol*. 2004;173:3392-3397.
- Schaper F, Rose-John S. Interleukin-6: Biology, signaling and strategies of blockade. *Cytokine Growth Factor Rev*. 2015;26:475-487.
- Ng TH, Britton GJ, Hill EV, Verhagen J, Burton BR, Wraith DC. Regulation of adaptive immunity; the role of interleukin-10. *Front Immunol*. 2013;4:129.
- Grewe M, Gyufko K, Krutmann J. Interleukin-10 production by cultured human keratinocytes: regulation by ultraviolet B and ultraviolet A radiation. *J Invest Dermatol*. 1995;104:3-6.
- Grone A. Keratinocytes and cytokines. *Vet Immunol Immunopathol*. 2002;88:1-12.
- Naik SM, Cannon G, Burbach GJ, et al. Human keratinocytes constitutively express interleukin-18 and secrete biologically active interleukin-18 after treatment with pro-inflammatory mediators and dinitrochlorobenzene. *J Invest Dermatol*. 1999;113:766-772.
- Cumberbatch M, Dearman RJ, Antonopoulos C, Groves RW, Kimber I. Interleukin (IL)-18 induces Langerhans cell migration by a tumour necrosis factor-α and IL-1β-dependent mechanism. *Immunology*. 2001;102:323-330.
- Trzeciak M, Glen J, Bandurski T, Sokolowska-Wojdylo M, Wilkowska A, Roszkiewicz J. Relationship between serum levels of interleukin-18, IgE and disease severity in patients with atopic dermatitis. *Clin Exp Dermatol*. 2011;36:728-732.
- Howell MD, Parker ML, Mustelin T, Ranade K. Past, present, and future for biologic intervention in atopic dermatitis. *Allergy*. 2015;70:887-896.
- Belkaid Y, Oldenhove G. Tuning microenvironments: induction of regulatory T cells by dendritic cells. *Immunity*. 2008;29:362-371.
- Wollenberg A, Kraft S, Hanau D, Bieber T. Immunomorphological and ultrastructural characterization of Langerhans cells and a novel, inflammatory dendritic epidermal cell (IDEC) population in lesional skin of atopic eczema. *J Invest Dermatol*. 1996;106:446-453.
- Bonness S, Bieber T. Molecular basis of atopic dermatitis. *Curr Opin Allergy Clin Immunol*. 2007;7:382-386.
- Hayashida S, Furusho N, Uchi H, et al. Are lifetime prevalence of impetigo, molluscum and herpes infection really increased in children having atopic dermatitis? *J Dermatol Sci*. 2010;60:173-178.
- Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity*. 2011;34:637-650.
- Skabytska Y, Wolbing F, Gunther C, et al. Cutaneous innate immune sensing of Toll-like receptor 2-6 ligands suppresses T cell immunity by inducing myeloid-derived suppressor cells. *Immunity*. 2014;41:762-775.
- Brunner PM, Emerson RO, Tipton C, et al. Nonlesional atopic dermatitis skin shares similar T-cell clones with lesional tissues. *Allergy*. 2017;72:2017-2025.

24. Travers JB, Kozman A, Mousdicas N, et al. Infected atopic dermatitis lesions contain pharmacologic amounts of lipoteichoic acid. *J Allergy Clin Immunol.* 2010;125:146-152.
25. Flacher V, Bouschbacher M, Verronese E, et al. Human Langerhans cells express a specific TLR profile and differentially respond to viruses and Gram-positive bacteria. *J Immunol.* 2006;177:7959-7967.
26. van der Aar AM, Sylva-Steenland RM, Bos JD, Kapsenberg ML, de Jong EC, Teunissen MB. Loss of TLR2, TLR4, and TLR5 on Langerhans cells abolishes bacterial recognition. *J Immunol.* 2007;178:1986-1990.
27. Akdis CA, Akdis M, Bieber T, et al. Diagnosis and treatment of atopic dermatitis in children and adults: European Academy of Allergology and Clinical Immunology/American Academy of Allergy, Asthma and Immunology/PRACTALL Consensus Report. *Allergy.* 2006;61:969-987.
28. Bieber T, Rieger A, Stingl G, Sander E, Wanek P, Strobel I. CD69, an early activation antigen on lymphocytes, is constitutively expressed by human epidermal Langerhans cells. *J Invest Dermatol.* 1992;98:771-776.
29. Wollenberg A, Mommaas M, Oppel T, Schottdorf EM, Gunther S, Moderer M. Expression and function of the mannose receptor CD206 on epidermal dendritic cells in inflammatory skin diseases. *J Invest Dermatol.* 2002;118:327-334.
30. Kupper TS, Horowitz M, Birchall N, et al. Hematopoietic, lymphopoietic, and proinflammatory cytokines produced by human and murine keratinocytes. *Ann N Y Acad Sci.* 1988;548:262-270.
31. Grice EA, Segre JA. The skin microbiome. *Nat Rev Microbiol.* 2011;9:244-253.
32. Lai Y, Cogen AL, Radek KA, et al. Activation of TLR2 by a small molecule produced by *Staphylococcus epidermidis* increases antimicrobial defense against bacterial skin infections. *J Invest Dermatol.* 2010;130:2211-2221.
33. Li D, Lei H, Li Z, Li H, Wang Y, Lai Y. A novel lipopeptide from skin commensal activates TLR2/CD36-p38 MAPK signaling to increase antibacterial defense against bacterial infection. *PLoS One.* 2013;8:e58288.
34. Liu H, Archer NK, Dillen CA, et al. *Staphylococcus aureus* epicutaneous exposure drives skin inflammation via IL-36-mediated T cell responses. *Cell Host Microbe.* 2017;22:653-666.
35. Kong HH, Oh J, Deming C, et al. Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. *Genome Res.* 2012;22:850-859.
36. Niebuhr M, Lutat C, Sigel S, Werfel T. Impaired TLR-2 expression and TLR-2-mediated cytokine secretion in macrophages from patients with atopic dermatitis. *Allergy.* 2009;64:1580-1587.
37. Tsybikov NN, Petrisheva I, Fefelova EV, Kuznik BI, Magen E. Expression of TLR2 and TLR4 on peripheral blood monocytes during exacerbation of atopic dermatitis. *Allergy Asthma Proc.* 2015;36:140-145.
38. Oh DY, Schumann RR, Hamann L, Neumann K, Worm M, Heine G. Association of the toll-like receptor 2 A-16934T promoter polymorphism with severe atopic dermatitis. *Allergy.* 2009;64:1608-1615.
39. Staeger H, Schaffner A, Schneemann M. Human toll-like receptors 2 and 4 are targets for deactivation of mononuclear phagocytes by interleukin-4. *Immunol Lett.* 2000;71:1-3.
40. Flo TH, Halaas O, Torp S, et al. Differential expression of Toll-like receptor 2 in human cells. *J Leukoc Biol.* 2001;69:474-481.
41. Wu Q, Martin RJ, Lafasto S, et al. Toll-like receptor 2 down-regulation in established mouse allergic lungs contributes to decreased mycoplasma clearance. *Am J Respir Crit Care Med.* 2008;177:720-729.
42. Bieber T. Atopic dermatitis. *N Engl J Med.* 2008;358:1483-1494.
43. Mueller T, Terada T, Rosenberg IM, Shibolet O, Podolsky DK. Th2 cytokines down-regulate TLR expression and function in human intestinal epithelial cells. *J Immunol.* 2006;176:5805-5814.
44. Jin H, Kumar L, Mathias C, et al. Toll-like receptor 2 is important for the T(H)1 response to cutaneous sensitization. *J Allergy Clin Immunol.* 2009;123:875-882.
45. Niebuhr M, Heratizadeh A, Wichmann K, Satzger I, Werfel T. Intrinsic alterations of pro-inflammatory mediators in unstimulated and TLR-2 stimulated keratinocytes from atopic dermatitis patients. *Exp Dermatol.* 2011;20:468-472.
46. Ahmad-Nejad P, Mrabet-Dahbi S, Breuer K, et al. The toll-like receptor 2 R753Q polymorphism defines a subgroup of patients with atopic dermatitis having severe phenotype. *J Allergy Clin Immunol.* 2004;113:565-567.
47. Pattabiraman G, Panchal R, Medvedev AE. The R753Q polymorphism in Toll-like receptor 2 (TLR2) attenuates innate immune responses to mycobacteria and impairs MyD88 adapter recruitment to TLR2. *J Biol Chem.* 2017;292:10685-10695.
48. Salpietro C, Rigoli L, Miraglia Del Giudice M, et al. TLR2 and TLR4 gene polymorphisms and atopic dermatitis in Italian children: a multicenter study. *Int J Immunopathol Pharmacol.* 2011;24(4 Suppl):33-40.
49. Can C, Yazicioglu M, Gurkan H, Tozkir H, Gorgulu A, Sut NH. Lack of association between toll-like receptor 2 polymorphisms (R753Q and A-16934T) and atopic dermatitis in children from Thrace Region of Turkey. *Balkan Med J.* 2017;34:232-238.
50. Mrabet-Dahbi S, Dalpke AH, Niebuhr M, et al. The Toll-like receptor 2 R753Q mutation modifies cytokine production and Toll-like receptor expression in atopic dermatitis. *J Allergy Clin Immunol.* 2008;121:1013-1019.
51. Mason LM, Wagemakers A, van 't Veer C, et al. *Borrelia burgdorferi* Induces TLR2-mediated migration of activated dendritic cells in an ex vivo human skin model. *PLoS One.* 2016;11:e0164040.
52. Kou K, Aihara M, Matsunaga T, et al. Association of serum interleukin-18 and other biomarkers with disease severity in adults with atopic dermatitis. *Arch Dermatol Res.* 2012;304:305-312.
53. Novak N, Valenta R, Bohle B, et al. FcεpsilonRI engagement of Langerhans cell-like dendritic cells and inflammatory dendritic epidermal cell-like dendritic cells induces chemotactic signals and different T-cell phenotypes in vitro. *J Allergy Clin Immunol.* 2004;113:949-957.
54. Syed AK, Reed TJ, Clark KL, Boles BR, Kahlenberg JM. *Staphylococcus aureus* phenol-soluble modulins stimulate the release of proinflammatory cytokines from keratinocytes and are required for induction of skin inflammation. *Infect Immun.* 2015;83:3428-3437.
55. Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H. Interleukin-18 regulates both Th1 and Th2 responses. *Annu Rev Immunol.* 2001;19:423-474.
56. Gros E, Bussmann C, Bieber T, Forster I, Novak N. Expression of chemokines and chemokine receptors in lesional and nonlesional upper skin of patients with atopic dermatitis. *J Allergy Clin Immunol.* 2009;124:753-760.
57. van den Bogaard EH, Tjabringa GS, Joosten I, et al. Crosstalk between keratinocytes and T cells in a 3D microenvironment: a model to study inflammatory skin diseases. *J Invest Dermatol.* 2014;134:719-727.
58. Wang JH, Doyle M, Manning BJ, Di Wu Q, Blankson S, Redmond HP. Induction of bacterial lipoprotein tolerance is associated with suppression of toll-like receptor 2 expression. *J Biol Chem.* 2002;277:36068-36075.
59. Albrecht V, Hofer TP, Foxwell B, Frankenberger M, Ziegler-Heitbrock L. Tolerance induced via TLR2 and TLR4 in human dendritic cells: role of IRAK-1. *BMC Immunol.* 2008;9:69.
60. Mandron M, Aries MF, Boralevi F, et al. Age-related differences in sensitivity of peripheral blood monocytes to lipopolysaccharide and *Staphylococcus aureus* toxin B in atopic dermatitis. *J Invest Dermatol.* 2008;128:882-889.

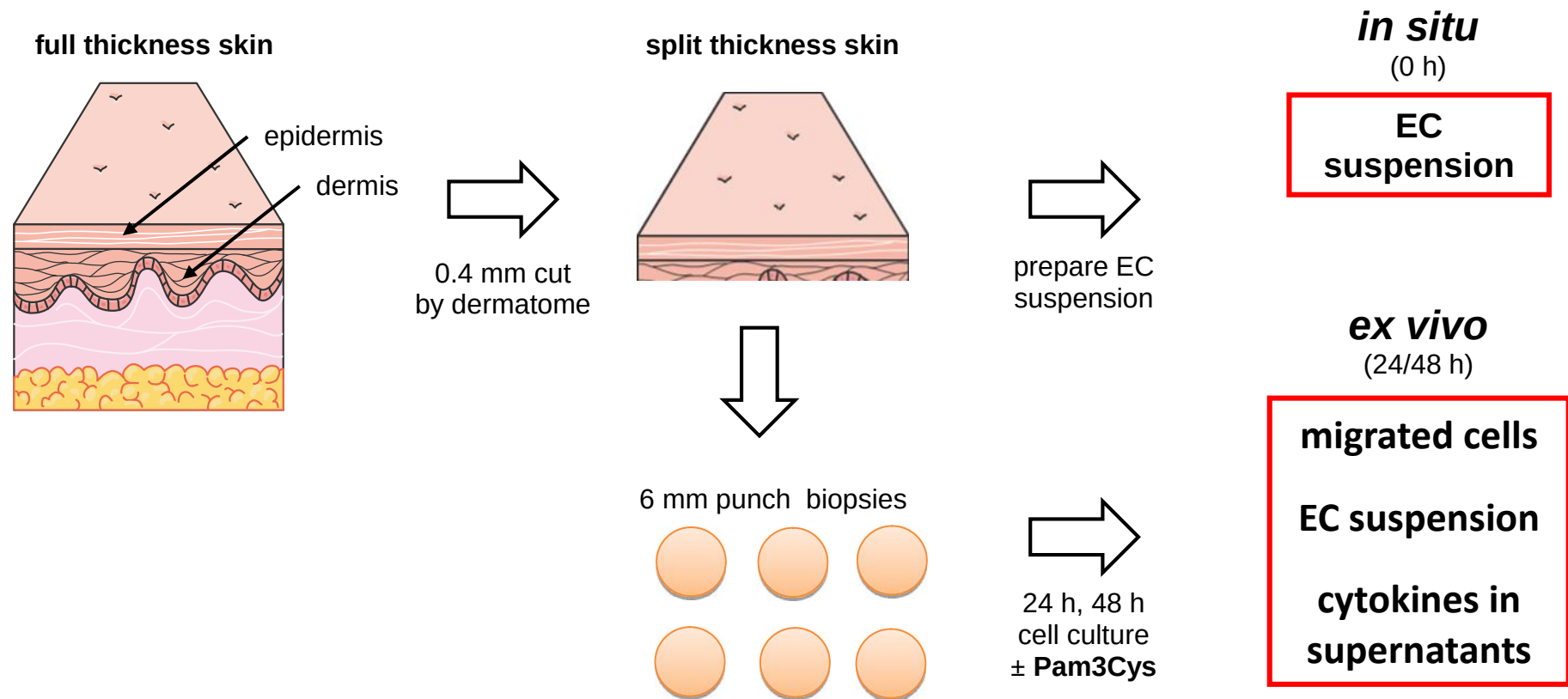
61. Hasannejad H, Takahashi R, Kimishima M, Hayakawa K, Shiohara T. Selective impairment of Toll-like receptor 2-mediated proinflammatory cytokine production by monocytes from patients with atopic dermatitis. *J Allergy Clin Immunol*. 2007;120:69-75.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

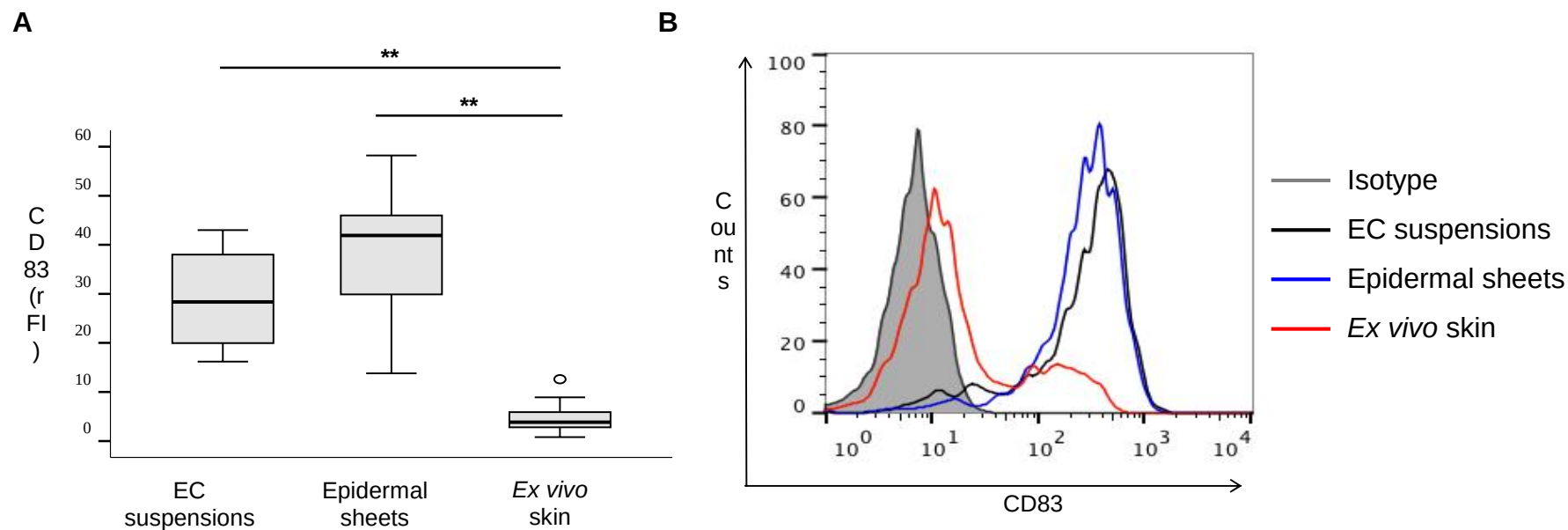
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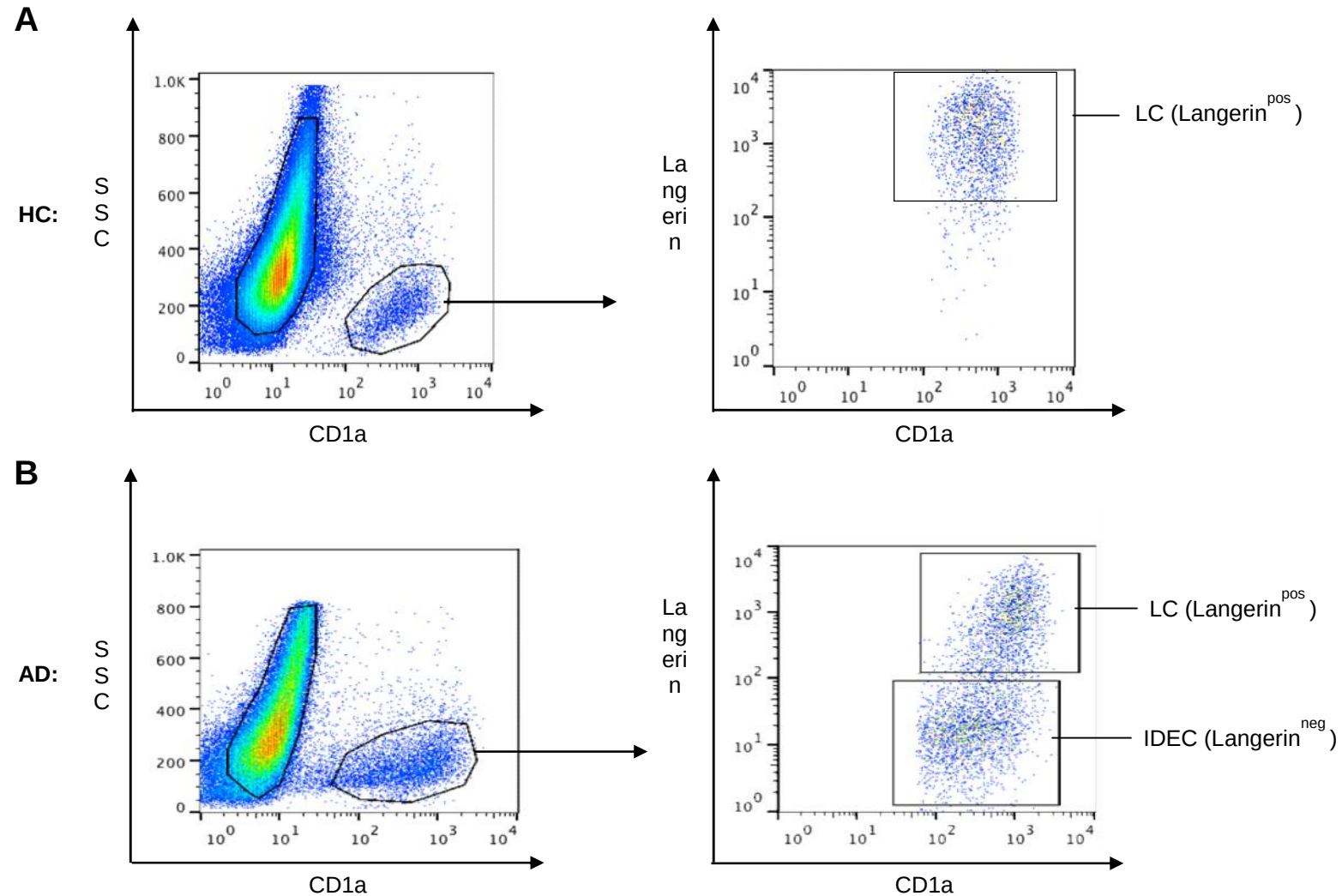
Online Repository Figure S1. Experimental overview.

AD skin was obtained by shave biopsies (average thickness of 0.4 mm) after local anesthesia. Healthy skin was obtained from patients immediately after plastic surgery using a dermatome. EC suspension was generated after 1 h trypsinization at 37 °C and analyzed afterwards by flow cytometry. For TLR2 ligation experiments, 6 mm punch biopsies were prepared from split thickness skin and incubated in medium with or without Pam3Cys for indicated time points. Migrated cells and supernatant were analyzed by flow cytometry or ELISA, respectively. EC suspension was prepared from the punch biopsies and analyzed by flow cytometry. Graphic was adapted from smart.servier.com and is licensed under CC BY 3.0 (<https://creativecommons.org/licenses/by/3.0/>).



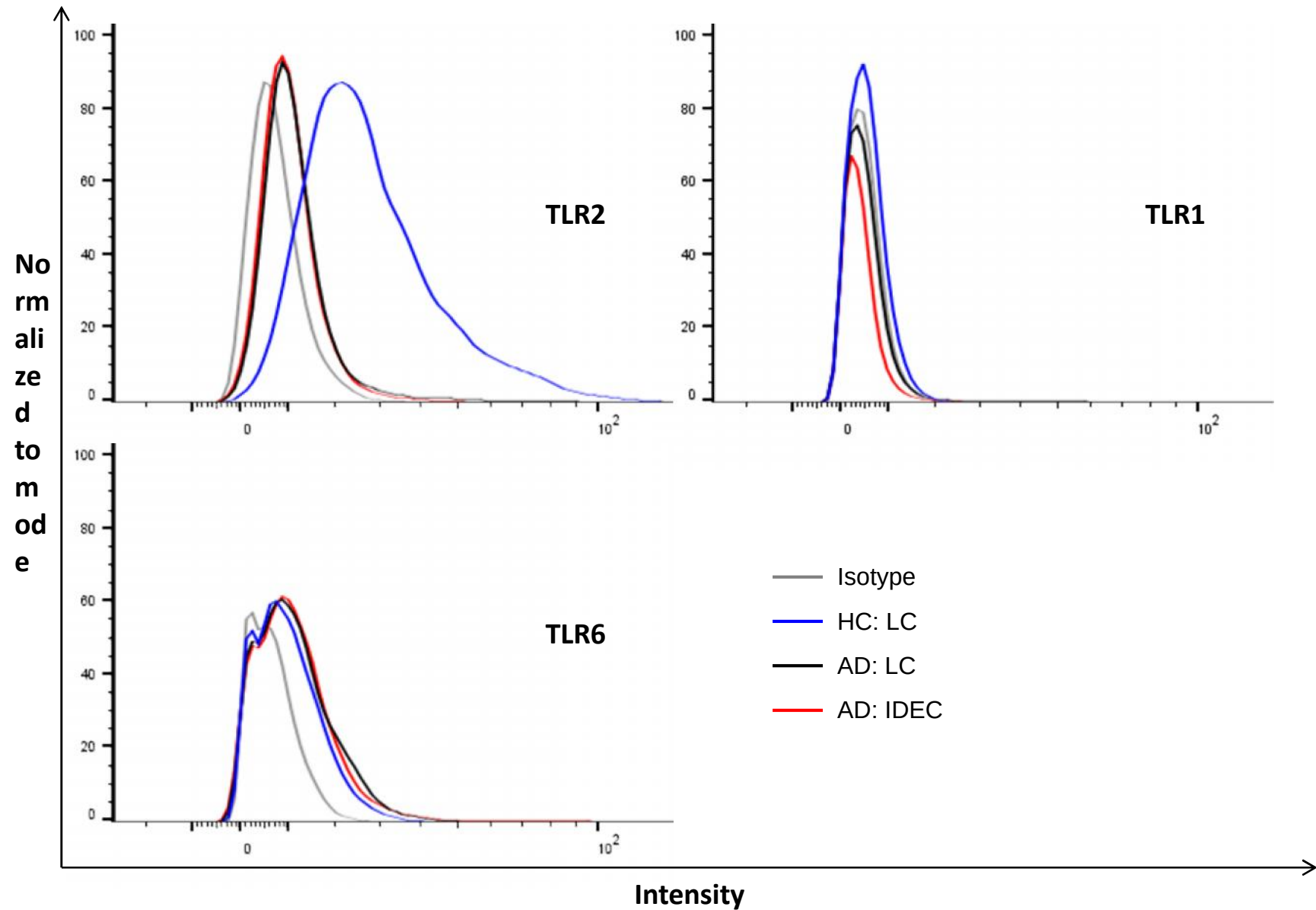
Online Repository Figure S2. *Ex vivo* skin culture system shows lowest spontaneous maturation of LC.

(A) EC suspensions (10^6 cells/ml, n=4), epidermal sheets (1 biopsy/well, n=5) and ex vivo skin (1 biopsy/well, n=12) from HC skin were cultured for 24 h without stimulation. After culture, the maturation marker CD83 of LC was compared between three different culture systems. A one-way ANOVA with Bonferroni post tests. **p<0.01 was used. **(B)** Representative histogram data of CD83 expression on LC are shown.



Online Repository Figure S3. Epidermal DC from AD skin display an additional Langerin^{neg} population compared to healthy skin.

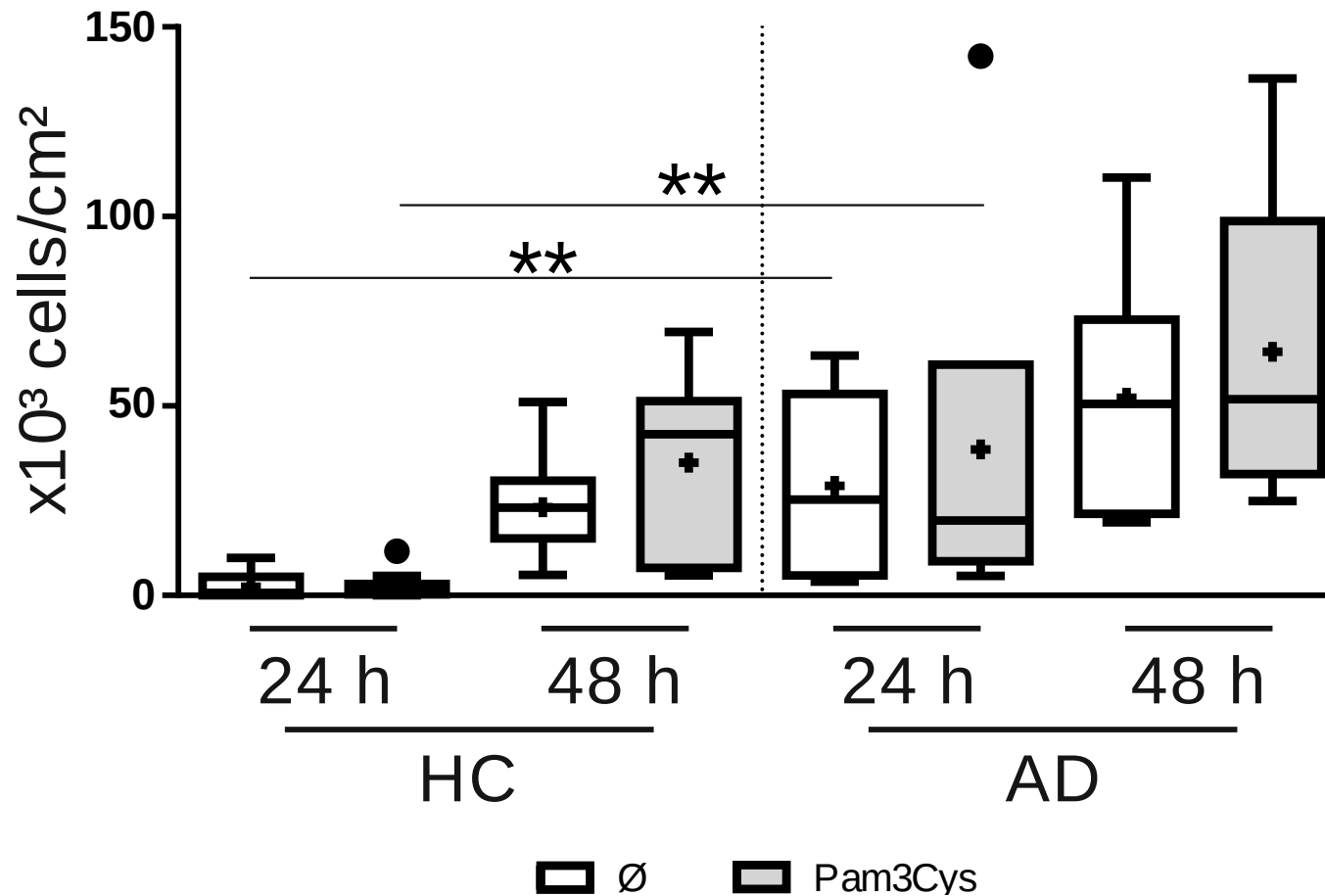
Representative dot plot of epidermal cell suspensions, which were prepared from healthy **(A)** or AD skin **(B)** and were stained with antibodies against CD1a and Langerin to discriminate between IDEC and LC in flow cytometry analysis.



Online Repository Figure S4. Freshly isolated epidermal DC from AD skin express less TLR2 *in situ* than those from healthy skin.

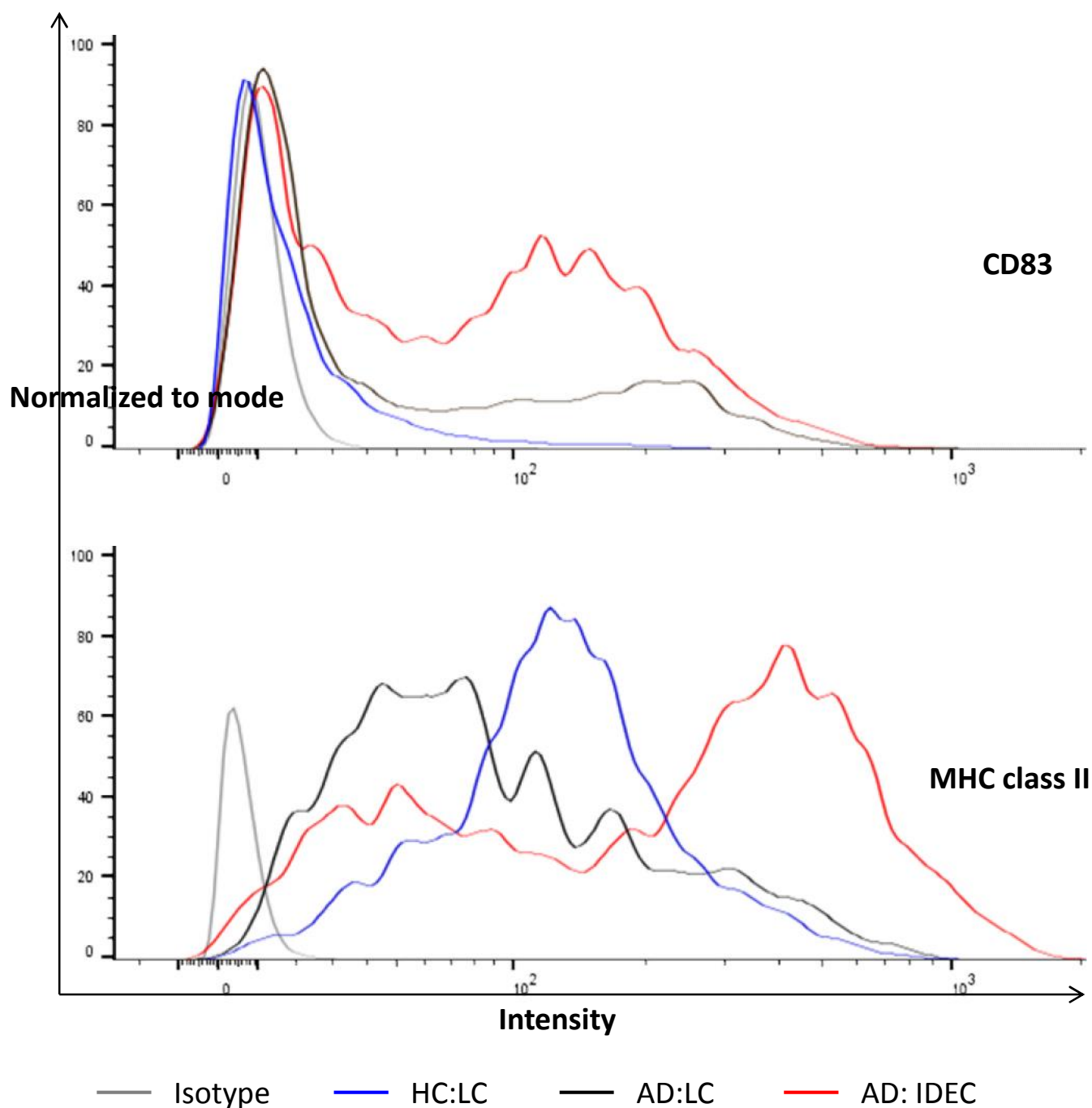
EC suspension was prepared from AD and healthy skin and stained with antibodies against CD1a and Langerin to discriminate between IDEC and LC in flow cytometry analysis. A representative overlay histogram of indicated TLRs is shown.

migrated CD1a



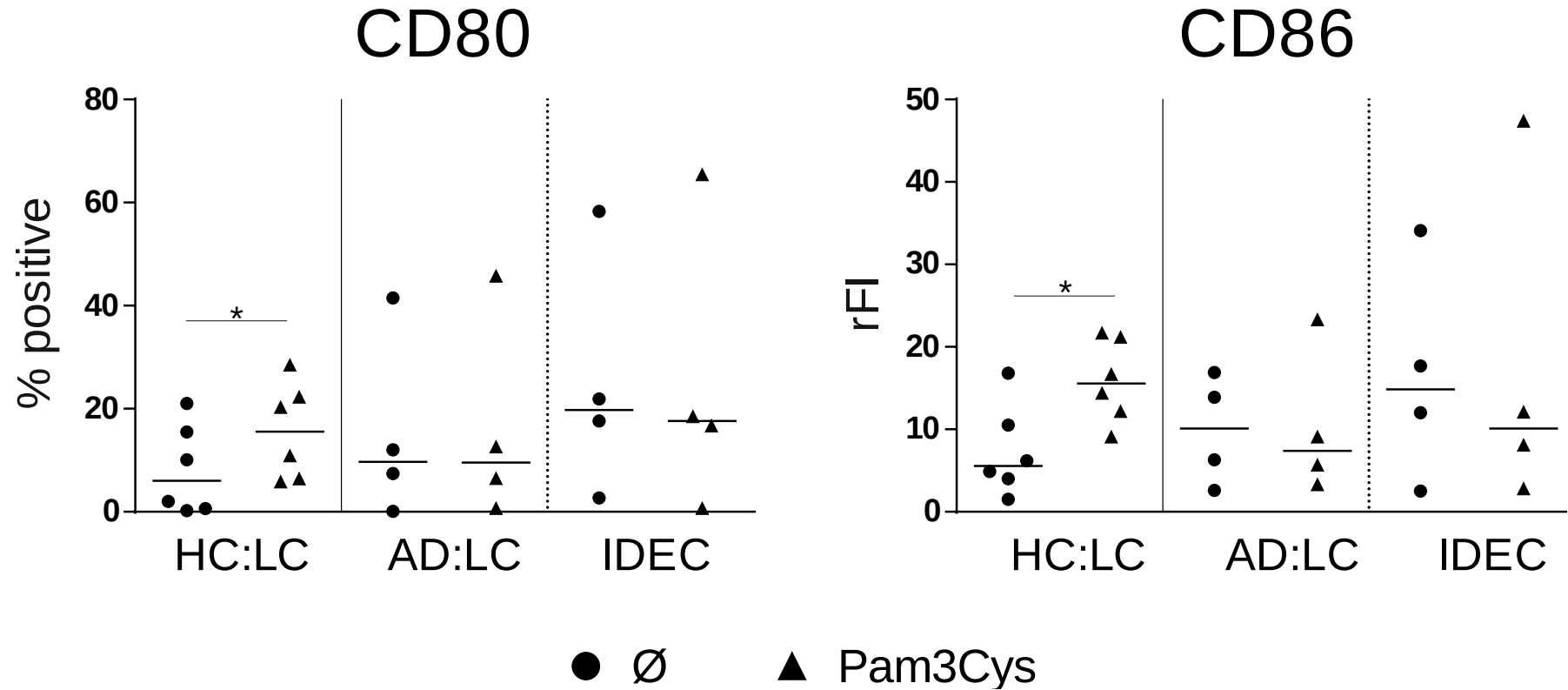
Online Repository Figure S5. TLR2 ligation enhances the emigration of LC from healthy, but not from AD skin.

Ex vivo human skin was cultured without (open bars) or with Pam3Cys (grey bars) for 24 h and 48 h, respectively. Emigrated cells were counted after the indicated time points, dead cells were excluded by trypan blue staining. The number of migrated cells was calculated based on flow cytometry analysis of CD1a expression of healthy (left panel, n = 10) and AD (right panel, n = 6). The Wilcoxon matched-pairs signed rank test was used for connected and the Mann-Whitney U test for independent samples.



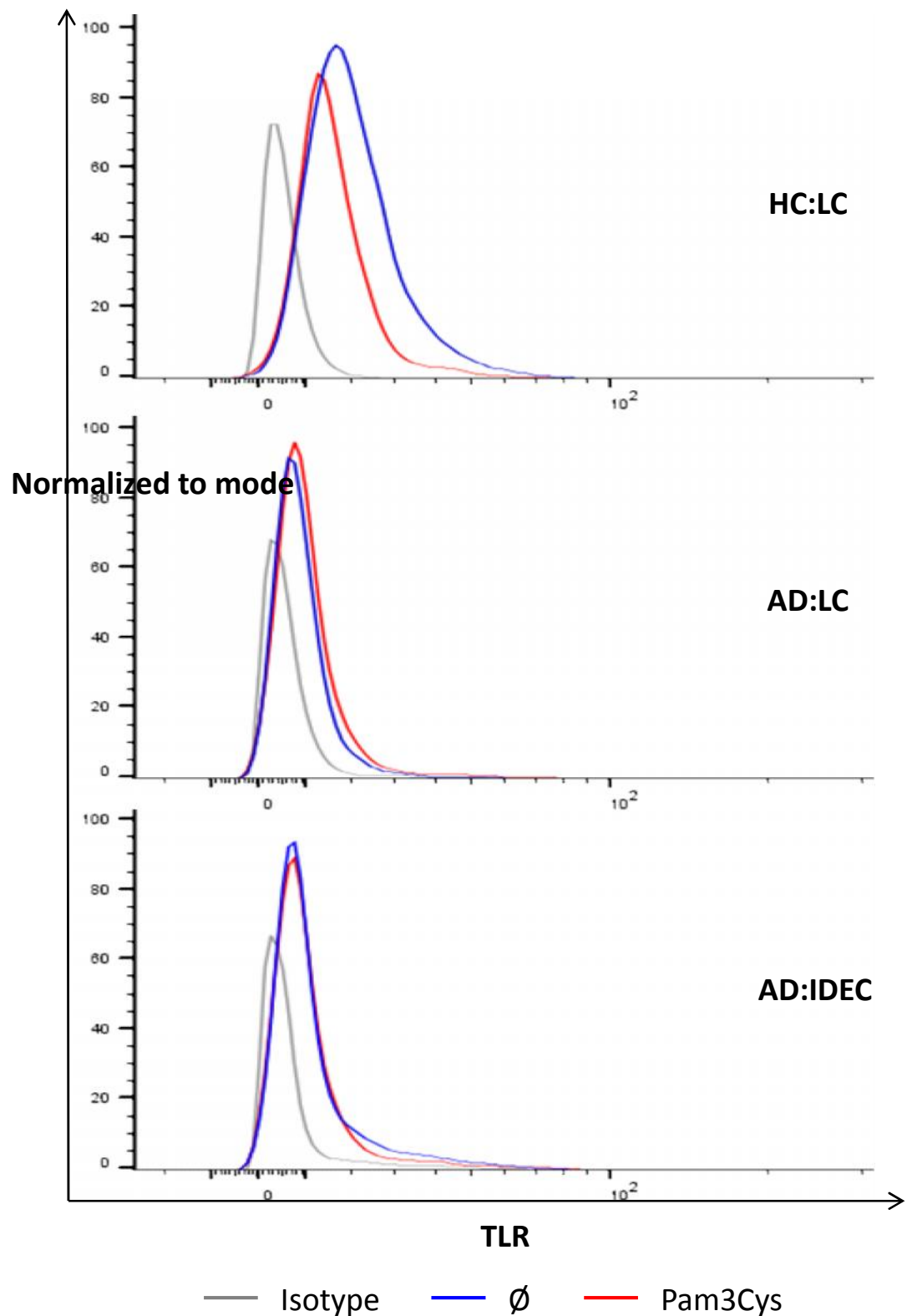
Online Repository Figure S6. Inflammatory dendritic epidermal cells but not Langerhans cells are activated *in situ*.

EC suspension was prepared from AD and healthy skin and stained with antibodies against CD1a and Langerin to discriminate between IDEC and LC in flow cytometry analysis. A representative overlay histogram of CD83 and MHC class II is shown for healthy (LC) and AD (LC and IDEC).



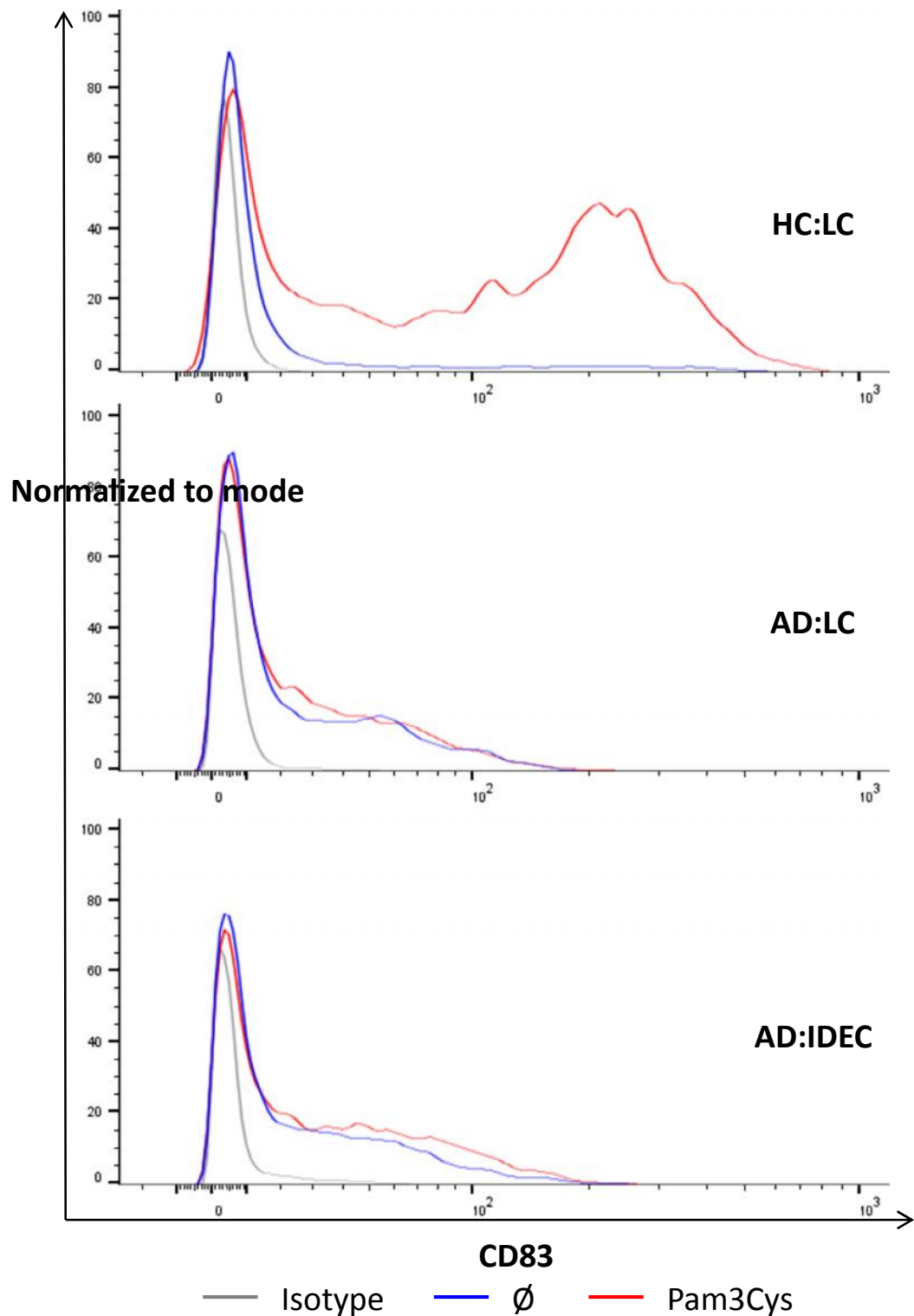
Online Repository Figure S7. TLR2 ligation enhances the maturation of LC in healthy but not in AD skin.

EC suspension was prepared from AD and healthy skin after 24 h cultivation with indicated stimulus and stained with antibodies against CD1a and Langerin to discriminate between IDEC and LC in flow cytometry analysis. Cell surface expression with indicated median of **(A)** CD80 (HC, n = 6; AD, n = 4) and **(B)** CD86 (HC, n = 6; AD, n = 4) was analyzed. The Wilcoxon matched-pairs signed rank test was used for connected samples.



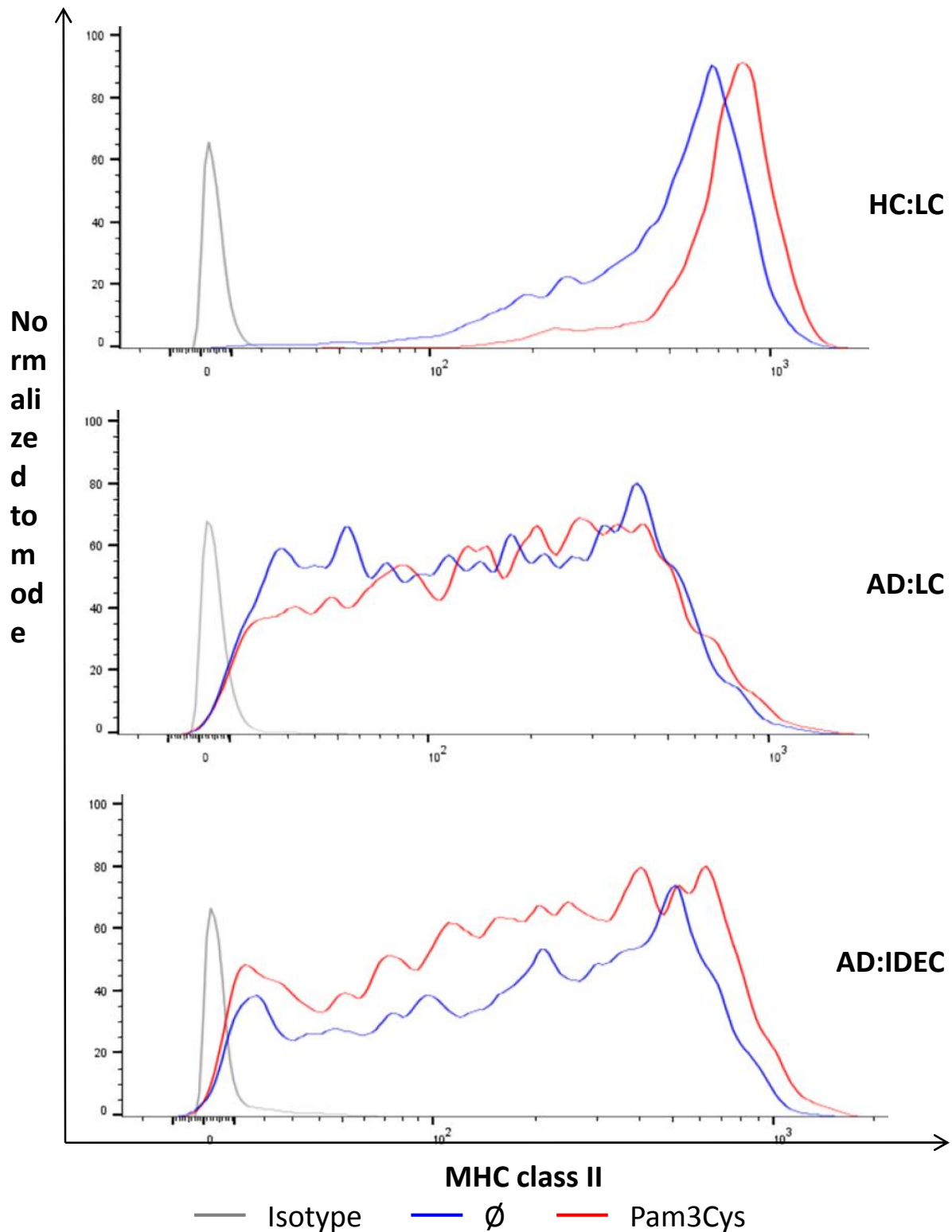
Online Repository Figure S8. TLR2 ligation reduces its expression on LC in healthy but not in AD skin.

EC suspension was prepared from AD and healthy skin after 24 h cultivation with or without Pam3Cys and stained with antibodies against CD1a and Langerin to discriminate between IDEC and LC in flow cytometry analysis. A representative overlay histogram of TLR2 is shown for healthy (LC) and AD (LC and IDEC).



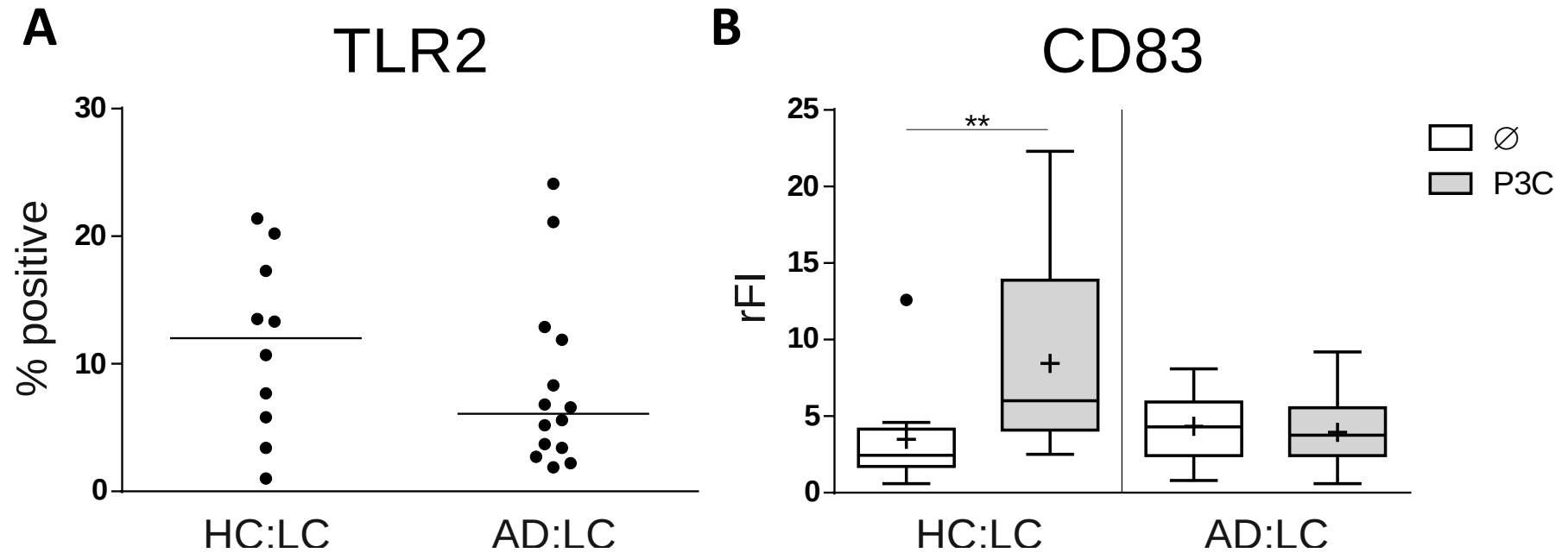
Online Repository Figure S9. TLR2 ligation enhances CD83 expression of LC in healthy but not in AD skin.

EC suspension was prepared from AD and healthy skin after 24 h cultivation with or without Pam3Cys and stained with antibodies against CD1a and Langerin to discriminate between IDEC and LC in flow cytometry analysis. A representative overlay histogram of CD83 is shown for healthy (LC) and AD (LC and IDEC).



Online Repository Figure S10. TLR2 ligation enhances MHC class II expression of LC in healthy but not in AD skin.

EC suspension was prepared from AD and healthy skin after 24 h cultivation with or without Pam3Cys and stained with antibodies against CD1a and Langerin to discriminate between IDEC and LC in flow cytometry analysis. A representative overlay histogram of MHC class II is shown for healthy (LC) and AD (LC and IDEC).



Online Repository Figure S11. Low TLR2 expressing healthy LC are still able to mature upon Pam3Cys ligation, while AD LC are not.

EC suspension was prepared from AD and healthy skin and stained with antibodies against CD1a and Langerin. TLR2 expression of freshly isolated LC was analysed using flow cytometry (A). The *ex vivo* human skin was cultured without (open bars) or with Pam3Cys (grey bars) for 24 h, followed by EC suspension generation and analysis of CD83 (B) using flow cytometry. The Wilcoxon matched-pairs signed rank test was used for statistical analysis (HC, n = 10; AD, n = 14).

OR Table S1. Antibodies used for flow cytometry.

* mAb W6/32 against MHC class I and mAb L243 against MHC class II were kindly provided by Dr. G. Moldenhauer (Heidelberg, Germany).

Antibodies	Conjugate	Manufacturer	Clone	Isotype	Dilution
TLR1		Santa Cruz	GD2.F4	IgG1	1:80
TLR2		IMGENEX	1030A5.138	IgG1	1:200
TLR6	FITC	IMGENEX	86B1153.2	IgG1-FITC	1:200
Langerin (CD207)	PE	Beckman Coulter	DCGM4	IgG1-PE	1:10
CD1a	APC	BD	HI149	IgG1-APC	1:10
CD80		BD	L307.4	IgG1	1:200
CD83		Beckman Coulter	HB15a	IgG2b	1:80
CD86		BD	IT2.2	IgG2b	1:200
CD206		BD	19.2	IgG1	1:200
MHC I		Provided*	W6/32	IgG2b	1:3
MHC II		Provided*	L243	IgG2b	1:4.3
CLIP		BD	CerCLIP	IgG1	1:200