Langerhans Cells Require MyD88-Dependent Signals for Candida albicans Response but Not for Contact Hypersensitivity or Migration

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Langerhans Cells Require MyD88-Dependent Signals for Candida albicans Response but Not for Contact Hypersensitivity or Migration

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Langerhans cells (LC) are a subset of skin-resident dendritic cells (DC) that reside in the epidermis as immature DC, where they acquire Ag. A key step in the life cycle of LC is their activation into mature DC in response to various stimuli, including epidermotropic sensitization with hapten and skin infection with Candida albicans. Mature LC migrate to the skin-draining LN, where they present Ag to CD4 T cells and modulate the adaptive immune response. LC migration is thought to require the direct action of IL-1β and IL-18 on LC. In addition, TLR ligands are present in C. albicans, and hapten sensitization produces endogenous TLR ligands. Both could contribute to LC activation. We generated Langerin-Cre MyD88fl mice in which LC are insensitive to IL-1 family members and most TLR ligands. LC migration in the steady state, after hapten sensitization and postinfection with C. albicans, was unaffected. Contact hypersensitivity in Langerin-Cre MyD88fl mice was similarly unaffected. Interestingly, in response to C. albicans infection, these mice displayed reduced proliferation of Ag-specific CD4 T cells and defective Th17 subset differentiation. Surface expression of costimulatory molecules was intact on LC, but expression of IL-1β, IL-6, and IL-23 was reduced. Thus, sensitivity to MyD88-dependent signals is not required for LC migration, but is required for the full activation and function of LC in the setting of fungal infection. The Journal of Immunology, 2012, 188: 000–000.

Peripheral dendritic cells (DC) reside in an immature state in nonlymphoid tissues, where they actively acquire Ag (1). In response to maturation stimuli, peripheral DC become activated and increase surface expression of costimulatory markers such as CD80 and CD86 as they migrate out of their resident tissue, through the lymphatics, to the regional draining lymph node (LN). Once in the LN, they present Ag acquired in the periphery to naive and memory T cells and thereby initiate adaptive immune responses. Peripheral DC that migrate during the steady state are thought to maintain peripheral self-tolerance.

Langerhans cells (LC) are a subset of skin-resident DC that forms a dense network in the epidermis (2). Although LC acquire Ag in the skin and migrate to the cutaneous LN (CLN), where they present Ag to T cells, their function remains somewhat controversial (3). Contact hypersensitivity (CHS) is a classic technique to assay cutaneous adaptive immune responses. Transgenic mice with an inducible or constitutive ablation of epidermal LC develop increased CHS to multiple haptens that results from the absence of LC during the afferent (i.e., sensitization) but not efferent (elicitation) phase (4–6). Data from other LC ablation mouse models find that CHS is reduced or unaffected by LC absence (7–9). A newer assay of LC function involves skin infection with Candida albicans. In response to C. albicans infection, footpad delayed-type hypersensitivity (DTH) responses are increased in the absence of LC. In addition, the differentiation of Ag-specific Th17 CD4+ T cells, but not Th1 or CD8+ CTL cells, is greatly diminished in LC-deficient mice (10). This phenotype is recapitulated in mice with a LC-specific ablation of MHC-II. Thus, LC that migrate to the CLN in response to C. albicans infection present Ag acquired in the skin to CD4 T cells and skew Th-phenotype differentiation, which ultimately determines the degree of the adaptive response.

A key step in the life cycle of LC is their activation from an immature DC in the epidermis to a fully mature DC in the CLN (2). LC migrate from the epidermis in response to many stimuli, including microbial products, chemical sensitizers (i.e., haptens), and UV light. LC migration after epicutaneous application of hapten is the best studied. Migration can be inhibited by in vivo blockade of TNF-α, IL-1α, IL-1β, or IL-18 with neutralizing Abs (11–13). Dermal injection of these cytokines is also sufficient to induce LC migration. In addition, LC migration is defective in IL-1R−/− and IL-18−/− mice (14–16).

Recently, a crucial role for TLR2 and TLR4 during the early afferent response of CHS was described (17). Mice lacking both TLR2 and TLR4 develop attenuated CHS. TLR2 and TLR4 appear to recognize endogenous ligands in the skin, such as low m.w. breakdown products of high m.w. hyaluronic acid that are generated by sensitization (17, 18). CHS also requires inflammasome-dependent production of active IL-1β and IL-18. Mice with defects in this pathway (i.e., P2X7−/−, ASC−/−, NALP3−/−, caspase1−/−, IL-1β−/−, IL-18−/−, and IL-1R−/− mice) all have defective CHS (14, 19–21). These data support a model in which...
hapten sensitization promotes elaboration of IL-1 family members and TLR agonists in the skin that induce activation and migration of skin-resident DC to the CLN, thereby initiating an adaptive antihapten response. Because C. albicans contains ligands for TLR2/4 and induces inflammasome activation, LC migration to C. albicans infection most likely occurs through a similar mechanism (22).

Although many of the factors involving LC migration have been identified, the cell type(s) that senses the presence of TLR agonists and responds to IL-1/IL-18 has not been definitively demonstrated. Because most DC subtypes respond to these factors, it is likely they act directly on LC to induce migration. To test this hypothesis, we generated Langerin-Cre MyD88-flox mice. MyD88 is a signaling adapter molecule downstream of the IL-1R, IL-18R, and most TLRs. MyD88−/− mice with global MyD88 defects fail to develop CHS (16). Langerin-Cre MyD88-flox mice have a selective ablation of MyD88 in LC that renders only these cells unresponsive. In this study, we examine the migratory capacity and function of MyD88-deficient LC in response to hapten sensitization and skin infection with C. albicans.

Materials and Methods

Mice

MyD88flox mice generated on a C57BL/6 background (23) were obtained and crossed with huLangerin-Cre YFPflox mice that had been backcrossed onto C57BL/6 for seven generations (24). Unless noted, littermate mice were used as controls. The YFP served as an endogenous reporter for Cre expression and, indirectly, MyD88 excision. HuLangerin-DTA (5) mice were employed as a positive control in CHS and DTH experiments. Texa Rag−/−CD4 TCR transgenic to I-Eκ to 60 bp (25) mice on CD90.1 C57BL/6 background were used for adoptive transfer experiments. The mice used in experiments were 6–10 wk old and sex matched. Mice were housed in microisolation cages and fed irradiated food and acidified water. The University of Minnesota institutional care and use committee approved all mouse protocols.

Antibodies

Fluorochrome-conjugated Abs to CD4, CD8, CD11b, CD11c, CD45.2, CD90.1, CD103, MHC-II, IFN-γ, IL-17A, and TLR2 were obtained from eBioscience (San Diego, CA). Abs to Langerin (clone 929F30.1) were obtained from Imgenex (San Diego, CA).

Flow cytometry

Single-cell suspensions from epidermis, dermis, and CLN were prepared and stained for skin DC subsets, as previously described (5). T cell cytokine expression was determined, as previously described (4). Samples were analyzed on LSR-II flow cytometers (BD Biosciences, San Jose, CA), and the resulting data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Cell sorting

Single-cell suspensions from epidermis and skin-draining LN were prepared using the same method as mentioned above. A FACSaria cell sorter was used to isolate LC (YFP+, MHC-II+) from epidermal cell suspensions. Cells from skin-draining LN were enriched and sorted, as described (10).

PCR and quantitative PCR

Epidermis-derived sorted LC underwent an overnight digestion step at 56°C to release DNA. PCR was completed with the DNA and the following primers: 5′-GGGAAATATGGACGACTCTCCCAGCAG-3′ and 5′-GACGTCATCTTCTCCCCCTGCG-3′. These primers allow for the discrimination of the wild-type (WT) allele, floxed allele, and deleted allele. The WT product was ~1600 bp, the floxed product was ~1800 bp, and the deleted allele product was ~400 bp. mRNA was extracted from sorted LC using a MiniPrep kit (Qiagen, Valencia, CA) and analyzed via quantitative PCR (qPCR) with TaqMan Gene Expression Assays and an ABI 7900HT (Applied Biosystems, Carlsbad, CA), as previously described (10). The data are presented as 2−ΔΔCt.

TLR2 agonist injection

Endotoxin-free Pam3CSK4 was purchased from Invivogen (San Diego, CA) and diluted to 1 mg/ml with sterile water, per manufacturer’s instructions. A total of 20 μl Pam3CSK4 or sterile water was injected into the dorsal ear pinnae. Four days later, the ears were excised and split in half. The ear sheets were processed into epidermal single-cell suspension, as previously described (5).

Epicutaneous cell labeling

Tetramethylrhodamine isothiocyanate (TRITC) was prepared by diluting 20 μg/μl stock solution to 1 μg/μl with 1:1 acetone and dibutyl phthalate. Abdomen hair was removed from mice with an electric clipper 1 d prior to painting. A total of 50 μl TRITC solution or vehicle was applied to the skin and allowed to dry before mice were returned to cage. Skin-draining LN were harvested 4 d later for flow cytometry analysis.

Contact hypersensitivity

Allergic contact dermatitis was induced with 2,4-dinitro-1-fluorobenzene (DNFB; Sigma-Aldrich, St. Louis, MO), as previously described (5). DFNB (0.5%) was applied to abdominal skin for sensitization, and 0.2% DFNB was used for the challenge. Five days passed between sensitization and challenge, and ear swelling was measured 1 d after challenge.

C. albicans skin infection

The dorsal flanks of mice were infected with recombinant C. albicans (designated Calb-Ag), as previously described (10). C. albicans was grown in yeast peptone adenine dextrose agar at 30°C until the OD600 measured between 1.5 and 2. Mice were anesthetized with ketamine/xylazine mixture (100/10 μg/kg body weight). Hair was removed from the skin with an electric clipper and Nair, per manufacturer’s instructions. The stratum corneum was removed with 220 grit sandpaper (3M, St. Paul, MN) before the application of 2 × 108 C. albicans in 50 μl sterile PBS.

Delayed-type hypersensitivity

The adaptive immune response was assessed after recombinant C. albicans infection via DTH, as previously described (10). A total of 105 heat-killed yeast cells was injected into the footpad of mice 7 d after skin infection. The footpad swelling was measured 1 d later. The specific DTH was calculated by subtracting the degree of swelling in PBS-treated mice from the degree of swelling in C. albicans-infected mice. The same recombinant strain of C. albicans was used for both skin infection and challenge.

Adaptive T cell transfer

Texa transgenic T cells were diluted and transferred, as previously described (10). Single-cell suspensions were created from the skin-draining and mesenteric LN of Texa mice. The cells were labeled with CFSE (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions, and resuspended in sterile PBS. A total of 3 × 105 cells, in 300 μl, was injected i.v. and was later detected in recipient mice by their expression of congenic CD90.1.

Statistical analysis

The significant differences between populations were calculated using the Mann–Whitney U test. All mRNA data comparisons used the Student unpaired, two-tailed t test.

Results

Generation of Langerin-Cre MyD88-flox mice

Langerin-Cre transgenic mice constitutively express Cre recombinase under the control of the human promoter for langerin (24). We have previously reported, using Langerin-Cre mice bred onto the YFP Cre reporter strain (Ros26.stop3-YFP), that Cre expression is restricted to epidermal DC and that floxed allele excision occurs efficiently. Other DC subsets, including Langerin+ CD103+ dermal DC, do not express Cre. We crossed Langerin-Cre Ros26.stop3-YFP mice with MyD88flox mice (23) and generated Langerin-Cre MyD88flox Rosa26.stop3-YFP mice, henceforth referred to as Langerin-Cre MyD88. As expected, all LC in the epidermis and CLN expressed YFP (data not shown). Although expression of YFP should be linked to excision of MyD88, we
confirmed the efficient excision of MyD88 by FACS sorting LC from Langerin-Cre MyD88 mice and isolating DNA for genomic PCR across the MyD88 locus. As expected, only a product from the 367-bp, fully excised MyD88 locus was visualized (LC, Fig. 1A). DNA isolated from the total LN of WT or Langerin-Cre MyD88 mice showed the expected 1543-bp product for the native MyD88 locus and 1761 bp for the unexcised MyD88 locus, respectively. As was observed with mice that constitutively lack LC, Langerin-Cre MyD88 mice displayed no gross abnormalities or evidence of spontaneous skin inflammation (5).

**MyD88-deficient LC migrate in the steady state**

To evaluate whether steady-state LC migration occurs through a MyD88-dependent process, we compared the number of resident epidermal LC in Langerin-Cre MyD88 and littermate control mice (WT). Flow cytometry of epidermal single-cell suspensions revealed a similar density of LC in both strains of mice (Fig. 1C). The number of LC, Langerin+ dermal DC (dDC) as Langerin+ dDC found in CLN was also similar in Langerin-Cre MyD88 and WT mice (Fig. 1D). These data are consistent with a previous report that the numbers of all Langerin+ DC in CLN are unaffected in MyD88−/− mice (26). LC in mice express TLR2 (Fig. 1B). To confirm that LC in Langerin-Cre MyD88 mice were functionally defective, we injected Pam3CSK4, a synthetic TLR1/2 agonist, or PBS into the dermis of both mouse strains (Fig. 1C). As expected, Pam3CSK4 induced migration of LC from the epidermis in WT but not Langerin-Cre MyD88 mice. Thus, activation via TLR1/2 is sufficient to induce LC migration, but steady-state LC migration does not require LC-intrinsic MyD88-dependent signaling.

**MyD88-deficient LC migrate in response to hapten**

Although LC migration to pharmacologic doses of TLR agonist required sensitivity to MyD88, we next examined whether MyD88 participates in the more physiologic assay of hapten-induced LC migration using TRITC. In addition to inducing inflammation, TRITC has the advantage of labeling those DC that are present in the skin at the time of painting, thereby allowing discrimination of DC that have migrated in response to hapten from those that migrated previously (27, 28).

TRITC was applied to cohorts of Langerin-Cre MyD88 and control Langerin-Cre YFP mice. After 4 d, CLN cells were analyzed via FACS, and four populations were identified, as follows:

**FIGURE 1.** MyD88-deficient LC migrate in the steady state. (A) DNA was isolated from LN of WT and MyD88-flox mice (left) and from LC sorted from Langerin-Cre MyD88 mice. PCR across the MyD88 locus is shown (expected products: endogenous locus, 1543 bp; unexcised floxed locus, 1761 bp; excised locus, 367 bp). (B) Single-cell suspensions of epidermis were gated on LC based on expression of MHC-II and stained for TLR2. Representative data from 10 individual mice are shown. (C) Langerin-Cre MyD88 mice (black) and littermate controls (WT, white) were injected in the ear with Pam 3CSK4, a synthetic TLR1/2 agonist, or PBS into the dermis of both mouse strains (Fig. 1C). As expected, Langerin-Cre MyD88 mice displayed no gross abnormalities or evidence of spontaneous skin inflammation (5).
TRITC+ YFP+ (LC that migrated in response to TRITC), TRITC+ YFP- (dDC that migrated in response to TRITC), TRITC- YFP+ (LC that migrated prior to TRITC application), and TRITC- YFP- cells (dDC that migrated prior to TRITC application) (Fig. 2A). We did not observe a difference in the number of these cell populations between Langerin-Cre MyD88 and Langerin-Cre YFP mice (Fig. 2B). Surface expression of DC activation markers such as CD40, CD80, and CD86 was highly expressed on migratory LC in CLN, but were equivalent in LC from both strains of mice (Fig. 2C). Similar results were obtained after application of the hapten DNFB (data not shown). Thus, the ability of LC to respond to MyD88-dependent signals is not required for migration or expression of activation markers in response to hapten-induced inflammation.

We have previously reported that Langerin-DTA mice that lack LC develop exaggerated CHS to numerous haptens (4, 5). To examine whether LC from Langerin-Cre MyD88 mice were functionally impaired, we sensitized Langerin-Cre Myd88 and littermate control mice with 0.5% DNFB. After 5 d, mice were challenged with 0.2% DNFB. As expected, the degree of ear swelling was exaggerated in Langerin-DTA mice (DTA), whereas ear swelling was unaffected in Langerin-Cre MyD88 mice (MyD88) (Fig. 2D). Thus, hapten-induced migration of LC and LC-mediated suppression of CHS does not require LC-intrinsic MyD88-dependent signaling.

MyD88-deficient LC migrate in response to C. albicans skin infection

Infection with the dimorphic fungus C. albicans has been shown in vivo to promote inflammasome activation and elaboration of IL-1β (22). C. albicans also contains ligands that are recognized by TLR2 and TLR4 (29). Because these are the same pathways that promote hapten-induced LC migration, we next examined LC migration in response to C. albicans skin infection. Four days postinfection, the number of LC, Langerin+ dermal DC, and Langerin- dermal DC in CLN of Langerin-Cre MyD88 and WT mice was compared. As was the case for hapten-induced migration, the numbers of migratory LC in Langerin-Cre MyD88 mice were unaltered (Fig. 3A). Surface expression of the activation markers CD40, CD80, and CD86 was similarly unaffected (Fig. 3B).

MyD88-deficient LC are functionally impaired

We have recently reported that Langerin-DTA mice that lack epidermal LC developed an exaggerated DTH response after skin infection with the dimorphic fungus C. albicans (22). C. albicans also contains ligands that are recognized by TLR2 and TLR4 (29). Because these are the same pathways that promote hapten-induced LC migration, we next examined LC migration in response to C. albicans skin infection. Four days postinfection, the number of LC, Langerin+ dermal DC, and Langerin- dermal DC in CLN of Langerin-Cre MyD88 and WT mice was compared. As was the case for hapten-induced migration, the numbers of migratory LC in Langerin-Cre MyD88 mice were unaltered (Fig. 3A). Surface expression of the activation markers CD40, CD80, and CD86 was similarly unaffected (Fig. 3B).

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infection with *C. albicans* (10). This was associated with modest decrease in proliferation of Ag-specific CD4 T cells and a near absence of Ag-specific Th17 cells. To test the functional importance of LC engagement with IL-1 family members and/or TLR agonists, we infected cohorts of Langerin-Cre MyD88, Langerin-DTA, and control mice with *C. albicans*. The footpad DTH response was examined 7 d later. As expected, Langerin-DTA mice developed an exaggerated specific DTH response. Interestingly, Langerin-Cre MyD88 mice developed an intermediate phenotype that was significantly increased above control, but was less than mice lacking LC (Fig. 4A). To examine the Ag-specific T cell response in more detail, we adoptively transferred CD90.1, TE2 peptide (10). Similar to Langerin-DTA mice, the expansion of TE2 cells was significantly reduced in Langerin-Cre MyD88 mice (Fig. 4B). The development of Th17 cells, as assayed by the expression of IL-17A, IL-17-F, and IL-22, was also significantly reduced compared with WT mice (Fig. 4C). Thus, MyD88-dependent signaling in LC was required for the development of an appropriate anti-Candi DA DTH response and differentiation of Ag-specific Th17 cells.

Because increased surface expression of costimulatory markers on LC after *C. albicans* infection was unaffected in Langerin-Cre MyD88 mice (Fig. 3B), we examined levels of cytokine expression. LC from Langerin-Cre MyD88 and control Langerin-Cre YFP mice were FACS sorted from CLN 4 d after *C. albicans* infection based on expression of CD11c<sup>+</sup>, MHC-II<sup>high</sup>, and YFP<sup>+</sup>. Levels of mRNA for IL-1β, IL-6, TGF-β, IL-12α, IL-12β, and IL-23α were examined by RT-qPCR (Fig. 4D). We observed a modest but significant decrease in IL-1β and IL-6 production in Langerin-Cre MyD88 mice. There was, however, a marked decreased expression of IL-12p40, a subunit shared between IL-12 and IL-23. Because LC do not produce message for IL-12α, these results indicate that LC from Langerin-Cre MyD88 mice have greatly reduced expression of IL-23. Generation of Th17 cells has been shown to depend on IL-1β, IL-6, IL-23, and TGF-β. The observed reduction of IL-1β, IL-6, and IL-23 expression in LC in the absence of MyD88-dependent signals is consistent with the reduced efficiency of Th17 development in response to infection with *C. albicans*.

**Discussion**

We have generated mice in which MyD88 is selectively ablated in LC. These mice show no deficit in LC migration during steady-state conditions, in response to hapten application, or as a result of skin infection with *C. albicans*. CHS is also functionally unaffected in these mice. In contrast, after skin infection with *C. albicans*, specific DTH responses, proliferation of Ag-specific CD4 T cells, differentiation of Th17 cells, and LC expression of Th17-promoting cytokines were all defective.

Skin application with haptons and infection with *C. albicans* have both been shown to activate the inflammasome and result in secretion of active IL-1β and IL-18. In addition, ligands for TLR2 and TLR4 are expressed by *C. albicans* and are generated in the skin in response to application of hapten (18, 29). The absence of MyD88 renders LC insensitive to IL-1β and IL-18. LC are also insensitive to many TLR agonists, except those binding TLR4 or TLR3. Thus, our observation that LC migration in Langerin-Cre MyD88 mice is intact demonstrates that direct activation of LC by MyD88-dependent TLR agonists, IL-1, or IL-18 is not an obligatory step for inflammation-induced LC migration. TLR4 is not expressed by LC (30), and TLR3 has not been reported to contribute to the in vivo hapten or *C. albicans* response. Thus, these data suggest a model in which IL-1 family members and TLR agonists act indirectly via cells other than LC, most likely keratinocytes, that then promote LC migration through a non-MyD88–dependent signal. TNF-α, a well-described keratinocyte-derived cytokine that induces LC migration through a MyD88-independent signaling pathway, is a possible candidate (11). Interestingly, dermal injection of *Pam3CSK₄*, a synthetic TLR1/2 agonist, produces modest MyD88-dependent LC migration. Thus, a strong MyD88-dependent signal when given in pharmacologic doses can induce LC migration. This raises the possibility that in response to physiologic stimuli, direct LC migration may occur, but be redundant, or that it may occur, but only with stimuli other than those we have examined.

CHS to epicutaneously applied hapten is the standard assay of the cutaneous adaptive immune response that is regulated by LC (3). Unlike Langerin-DTA mice that lack LC and develop exaggerated CHS, Langerin-Cre MyD88 mice manifest normal CHS. Thus, hapten-mediated release of IL-1β and production of endogenous TLR ligands, which are all required for optimal CHS, do not directly affect LC function and must act on other cell types. Expression of MyD88 in radioresistant cells has been shown to be required for CHS (16). Our data show that this dependence must reside in a cell type other than LC.

In the setting of skin infection with *C. albicans*, MyD88-deficient and sufficient LC efficiently migrated to skin-draining LN and increased surface expression of costimulatory molecules. In contrast, Ag-specific T cell proliferation was decreased, and differentiation of Th17 cells was severely impacted in Langerin-Cre MyD88 mice. Thus, indirect activation of LC is sufficient for migration, but does not result in the full activation required for appropriate T cell activation. We also observed that MyD88-deficient LC expressed reduced message for IL-1β, IL-6, and IL-23, which all participate in the development of Th17 cells. These data are consistent with earlier reports showing that DC require direct engagement with TLR agonists to elaborate the cytokines that are required for a productive T cell response (31). Thus, the commonly used assays of LC maturation, migration and expression costimulatory markers, need to be coupled with an examination of LC-derived cytokines to accurately determine the activation state of LC.

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**Disclosures**

The authors have no financial conflicts of interest.

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