Candida albicans Morphology and Dendritic Cell Subsets Determine T Helper Cell Differentiation

Highlights

- C. albicans yeast, but not filamentous forms, are required for Th17 cell responses
- Th17 cell induction requires LC-derived IL-6 and Dectin-1 ligation
- Absent Dectin-1 ligation by pseudo-hyphae prevents Th17 cell induction by CD11b+ dDCs
- Th17 cells provide cutaneous protection and Th1 cells provide systemic protection

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In Brief

Candida albicans is a dimorphic fungus responsible for chronic mucocutaneous and systemic infections. Kaplan and colleagues demonstrate in a skin infection model that yeast forms induce skin-protective Th17 cell responses by driving Langerhans cell expression of interleukin-6. Filamentous forms induce Th1 cell responses that provide protection from systemic infection.
Candida albicans Morphology and Dendritic Cell Subsets Determine T Helper Cell Differentiation

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SUMMARY

Candida albicans is a dimorphic fungus responsible for chronic mucocutaneous and systemic infections. Mucocutaneous immunity to C. albicans requires T helper 17 (Th17) cell differentiation that is thought to depend on recognition of filamentous C. albicans. Systemic immunity is considered T cell independent. Using a murine skin infection model, we compared T helper cell responses to yeast and filamentous C. albicans. We found that only yeast induced Th17 cell responses through a mechanism that required Dectin-1-mediated expression of interleukin-6 (IL-6) by Langerhans cells. Filamentous forms induced Th1 without Th17 cell responses due to the absence of Dectin-1 ligation. Notably, Th17 cell responses provided protection against cutaneous infection while Th1 cell responses provided protection against systemic infection. Thus, C. albicans morphology drives distinct T helper cell responses that provide tissue-specific protection. These findings provide insight into compartmentalization of Th cell responses and C. albicans pathogenesis and have critical implications for vaccine strategies.

INTRODUCTION

Candida albicans is a human commensal and a common mucosal and systemic pathogen in the setting of immunosuppression. Chronic mucocutaneous candidiasis (CMC) occurs in the absence of innate sources of interleukin-17 (IL-17) and IL-22 as well as in the setting of ineffective T helper 17 (Th17) cell-type immunity in both mice and humans (e.g., HIV/AIDS or hyper IgE syndrome) (Hernández-Santos et al., 2013; McDonald, 2012; Murray et al., 1985). CMC is also associated with mutations in the fungal recognition C-type lectin receptor Dectin-1 or its downstream signaling molecules (Ferwerda et al., 2009; Glocker et al., 2009). In contrast, systemic candidiasis predominantly occurs in the clinical setting of neutropenia and in mice with innate immune defects (e.g., Il6−/−, TGF-β−/−) resulting in ineffective neutrophil activation but does not occur in the absence of Th17-cell-associated or adaptive immunity (Bär et al., 2014; Horn et al., 2009; van de Veerdonk et al., 2010; van Enckevort et al., 1999).

C. albicans is dimorphic and grows as yeast at 30°C and as a filamentous or hyphal form at 37°C. On the stratum corneum of the skin, C. albicans exists as budding yeasts. Pathogenic C. albicans in the dermis and systemic organs exists predominantly as pseudo-hyphae (Gow et al., 2012). C. albicans mutants that are unable to form filaments fail to establish robust infections, suggesting that the yeast-to-hyphal transition is required for virulence (Lo et al., 1997). This transition has also been suggested to be required for the development of anti-Candida Th17 cell responses, thereby allowing for discrimination between commensal and invasive C. albicans (Gow et al., 2012). In vitro, C. albicans pseudo-hyphae induce Dectin-1-mediated Th17 cell differentiation (Cheng et al., 2011). C. albicans pseudo-hyphae, however, have also been reported to promote Th1 and Th2 cell differentiation in vitro (d’Ostiani et al., 2000; van der Graaf et al., 2005). Importantly, T helper (Th) cell responses in vivo to yeast and filamentous forms of C. albicans and the ability of these adaptive responses to provide protection remain unclear.

In the skin, there are at least three well-defined subsets of dendritic cells (DCs): epidermal Langerhans cells (LCs), CD103+ dermal DC (dDCs), and CD11b+ dDCs (Kaplan, 2010). These DC subsets migrate from the skin into regional lymph nodes during infection where they present antigen to naive T cells and secrete cytokines that determine Th cell differentiation (Reis e Sousa, 2004). During epicutaneous infection with C. albicans, CD103+ dDCs generate IL-12 and are required for differentiation of Th1 cells (Igyártó et al., 2011). LCs are required for Th17 cell differentiation and express high amounts of Th17-cell-inducing cytokines IL-1β, transforming growth factor-β (TGF-β), and
IL-6. The importance of individual cytokines in generating Th17 cells in vivo is controversial and varies by tissue. It has been demonstrated that IL-1β and IL-6 are both necessary for Th17 cell differentiation in peripheral tissues including skin (Hu et al., 2011). Th17 cell induction in the spleen is independent of IL-6 but not IL-1β. In the intestines, several studies have confirmed an IL-1β dependence for Th17 cell development although the requirement for IL-6 remains controversial (Hu et al., 2011; Persson et al., 2013; Shaw et al., 2012).

CD11b+ DCs make up the majority of the skin migratory DCs and drive Th2 cell differentiation in the setting of dermal papain injection or parasitic infection (Gao et al., 2013; Kumamoto et al., 2013). In the setting of C. albicans infection, this DC subset generates high amounts of IL-1β and to a lesser extent IL-6 and IL-12 (Igyártó et al., 2011). Their ability, however, to promote Th cell differentiation other than Th2 cell differentiation remains unexplored (Kumamoto et al., 2013). Based on the functional differences between skin DC subsets and the transition of C. albicans from yeast to filamentous forms during epidermal invasion, we hypothesized that DC subsets and C. albicans morphology together determine Th cell differentiation.

Because of the requirement for immunosuppression to establish robust and consistent oropharyngeal C. albicans infection, we have focused on epicutaneous C. albicans infection. We found that infection with C. albicans yeast but not pseudo-hyphae was capable of inducing Th17 cell responses through a mechanism that required interaction with LCs, engagement of Dectin-1, and LC-derived IL-6. C. albicans in the dermis failed to induce Th17 cell differentiation despite the expression of Dectin-1 on CD11b+ DCs due to the absence of Dectin-1 ligation by pseudo-hyphae that are the dominant morphology at that site. Finally, we demonstrated that Th17 but not Th1 cells were protective against secondary cutaneous infections whereas Th1 but not Th17 cells were protective against secondary systemic infections. Thus, C. albicans morphology and skin DC subsets drive distinct Th cell responses that provide protection from either cutaneous or systemic infections.

RESULTS

Distinct T Helper Cell Responses Mediated by C. albicans Morphology

We have previously generated recombinant C. albicans derived from the standard SC5314 strain that expresses the peptides 2W1S and Ez, under the ubiquitous Eno1 promoter (Eno1-Ag). Using a TEx CD4+ T cell adoptive transfer system, we showed that mice that express diphertheria toxin fragment A (DTA) under a human Langerin (huLangerin) promoter with a constitutive absence of LCs (henceforth referred to as LC−) failed to generate antigen-specific Th17 cells (Igyártó et al., 2011). To exclude the possibility that this phenotype resulted from a chronic absence of LCs, we repeated these experiments with mice that express the diphertheria toxin receptor (DTR) within huLangerin (referred to has huLangerin-DTR mice) that allow for inducible ablation of LCs (Bobr et al., 2010). As expected, ablation of LCs just prior to Eno1-Ag infection also prevented efficient Th17 cell differentiation (Figure S1A). We next examined the responses of endogenous antigen-specific CD4+ T cells to C. albicans by using I-Ab:p2W1S tetramer staining 8 days after infection. Expansion of p2W1S-specific cells was equivalent in control and LC-deficient mice (Figure 1A). Expansion of cells producing IL-17A but not interferon-γ (IFN-γ), however, was significantly reduced in LC-deficient mice (Figure 1B). Thus, consistent with our earlier observations using adoptive transfer of T cell receptor (TCR) transgenic T cells specific for Ez (TEx), LCs are required for efficient differentiation of endogenous naive CD4+ T cells into Th17 cells.

To determine whether recognition by LCs of yeast or filamentous forms of C. albicans affects Th cell differentiation, we selectively targeted antigen to LCs by i.p. injection of 1.0 μg anti-huLangerin mAb-Ez conjugates (2G3-Ez) in human Langerin transgenic mice (huLangerin) (Figure S1B; Flamar et al., 2012; Igyártó et al., 2011; Kaplan et al., 2012). HuLangerin is expressed exclusively by LCs in these mice, resulting in efficient and LC-selective acquisition of 2G3-Ez. As a source of adjuvant, we epicutaneously infected 2G3-Ez-targeted huLangerin mice with wild-type SC5314 as well as cph1/cph1 and tup1/tup1 mutants derived from SC5314 that are locked into yeast or hyphal forms, respectively (Braun and Johnson, 1997; Liu et al., 1994). Expansion of adoptively transferred TEx cells was equivalent in mice infected with all three strains (Figure 1C). Notably, Th17 cell differentiation was intact in the mice infected with the yeast-locked cph1 mutant but was absent in those infected with the filament-locked tup1 mutant, suggesting that LC-mediated Th17 cell differentiation requires the presence of C. albicans yeast forms (Figure 1D).

To determine the effect of C. albicans morphology under conditions in which antigen presentation is not limited to LCs, we next generated recombinant C. albicans in which expression of a fusion protein of GFP with the antigens Ez and 2W1S was driven by the promoter for the pseudo-hyphae-specific gene Hwp1 (Hwp1-Ag) (Staab et al., 1999). Unlike Eno1-Ag where GFP expression was present in all C. albicans morphologies, expression of GFP by Hwp1-Ag was absent in yeast and observed only for filamentous forms (Figures 1E and 2A–S2E). Both Eno1-Ag and Hwp1-Ag strains demonstrated similar virulence in vivo after epicutaneous or intravenous infection (Figure S2F and S2G). Epicutaneous infection of WT mice with Eno1-Ag and Hwp1-Ag induced similar antigen-specific CD4+ T cells expansion and Th1 cell differentiation (Figure 1F). Notably, mice infected with Hwp1-Ag failed to efficiently induce Th17-cell-associated responses (Figure 1G). Thus, LCs and C. albicans yeast but not pseudo-hyphae are required for optimal Th17 cell differentiation.

Th17 Cell Differentiation Requires LC-Derived IL-6

In vitro, Th17 cell differentiation of naive cells requires combinations of IL-1β, IL-6, IL-23, and TGF-β (Chung et al., 2009; Ivanov et al., 2006; Zúñiga et al., 2013). The requirement for IL-1β and IL-6 in vivo differs based on the site of immunization (Hu et al., 2011; Shaw et al., 2012). To determine which cytokines are responsible for LC-mediated Th17 cell induction, we first compared responses in IL-1β- and IL-6-deficient mice. We found that both IL-1β and IL-6 deficiency resulted in diminished Th17 cell responses (Figures 2A and 2B). Notably, Th17 cells were entirely absent in IL6−/− mice and was associated with a reciprocal increase in Th1 cells (Figures 2B and 2C).

Because LCs express of IL-1β, TGF-β, and IL-6 during C. albicans infection (Igyártó et al., 2011), we next sought to
determine which cytokines were required for Th17 cell differentiation. To generate LC-specific ablation of TGF-β1, we used huLangerin-creER-T2 Tgfβ1f/f mice that efficiently and selectively excise TGF-β1 in LCs after administration of tamoxifen (Bobr et al., 2012). We have previously reported that ablation of TGF-β1 in LCs results in a gradual spontaneous migration of LCs out of the epidermis over the course of weeks. To avoid this potential confounding factor, we infected mice on day 5 after tamoxifen administration, a time point when TGF-β1 has been efficiently excised but LCs have not begun to migrate in large numbers. As controls, we also infected tamoxifen-treated huLangerin-creER-T2 YFP and huLangerin-creER-T2 Tgfbr2f/f mice, which maintain TGF-β expression but also have increased homeostatic migration (Bobr et al., 2012). Antigen-specific T cell expansion and Th cell phenotype differentiation was equivalent in all three strains, demonstrating that LC-derived TGF-β is not required for Th17 cell differentiation (Figures 3A and 3B).

To generate LC-specific ablation of IL-1β and IL-6, we generated WT→Il1b−/− and WT→Il6−/− bone marrow chimeras. LCs are the only radio-resistant antigen-presenting cells (APCs) in the skin and remained of cytokine-deficient host origin while the hematopoietic system achieved 90%–96% chimerism (Figures S3A–S3C). Infected WT→Il1b−/− chimeras developed cell proliferation and Th cell differentiation similar to control WT→WT mice, indicating that LC-derived IL-1β is not required for Th17 cell induction (Figures 3C and 3D). In contrast, Th17 cell

Figure 1. Candida albicans Yeast Drives Th17 Cell Differentiation In Vivo
(A) The number of 2W1S-specific CD4+ T cells in six skin draining lymph nodes (axillary, brachial, inguinal) and spleen 8 days after mock (Neg) or skin infection with Eno1-Ag in wild-type (WT) or LC-deficient (LC−) mice is shown.
(B) Percentage of PMA-and-ionomycin-stimulated 2W1S-specific cells in WT (white) or LC− (black) mice expressing the indicated cytokines.
(C) Antigen was targeted to huLangerin transgenic mice by i.p injection of 1.0 μg 2G3-Ea. Adjuvant was provided by skin infection with wild-type, cph1/cph1, or tup1/tup1 strains of C. albicans. TEa numbers were assessed in the skin draining lymph nodes of mice 4 days after infection. Neg refers to unimmunized mice.
(D) The expression of IFN-γ and IL-17A in PMA-and-ionomycin-stimulated Ea-specific CD4+ TEa cells from (C) is shown.
(E) GFP expression in Eno1-Ag or Hwp1-Ag C. albicans under yeast or filamentous growth conditions was determined by immunofluorescence.
(F and G) TEa expansion (F) and cytokine production (G) 4 days after infection of WT mice with Eno1-Ag (white) or Hwp1-Ag (black) or naive (Neg). Each symbol represents data from an individual animal. Scales represents mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. Data are representative of two to three independent experiments. See also Figures S1 and S2.
differentiation was reduced in WT → Il6−/− chimeras, indicating that LC-derived IL-6 is required for Th17 cells (Figures 3E and 3F). Moreover, Th17 cell differentiation was intact in Il6−/− WT mice in which LCs were the only IL-6-sufficient hematopoietic cell type (Figure 3H). Thus, IL-6 derived from LCs is necessary for Th17 cell induction during skin infection.

**LC-Derived IL-6 Requires Dectin-1 Engagement**

A subset of patients with chronic mucocutaneous candidiasis (CMC) have mutations in the transcription factor STAT3, which acts downstream of the IL-6 receptor (McDonald, 2012). Patients with Dectin-1 polymorphisms also suffer from CMC and have decreased IL-6 production by their peripheral blood mononuclear cells (PBMCs) (Ferwerda et al., 2009). In addition, budding yeast engages Dectin-1 whereas yeast but not pseudo-hyphae trigger IL-6 secretion in human PBMCs (Armitage et al., 2011). Because Th17 cell differentiation depends on LC-derived IL-6, we next sought to determine whether Th17 cell differentiation required engagement of Dectin-1 on LCs. We took advantage of a clinical isolate, s20175.016, that has similar virulence as SC5314 but its immune recognition is independent of Dectin-1 (Figure S4A and S4B; Marakalala et al., 2013). Antigen was targeted to LCs in huLangerin mice using 2G3-Eα followed by infection with SC5314 or s20175.016. As expected, CD4+ T cell expansion and Th1 cell differentiation were comparable between the two experimental groups but Th17 cell differentiation was reduced in mice infected with s20175.016 (Figures 4A and 4B). Analysis of LCs isolated from the skin draining lymph nodes 3 days after infection revealed a large reduction in IL-6 production in mice infected with s20175.016 compared with SC5314 (Figures 4C and S5). Moreover, intradermal injection of 1 mg recombinant IL-6 at the time of infection restored Th17 cell differentiation (Figure 4D). Finally, we directly explored the necessity of Dectin-1 signaling by comparing the host response to C. albicans infection in Dectin-1 deficient (Clec7a−/−) mice. Whereas WT and Clec7a−/− mice strains exhibited similar...
T cell expansion, Dectin-1-deficient host demonstrated diminished Th17 cell responses (Figures 4E and 4F). Thus Th17 cell differentiation requires engagement of Dectin-1 on LCs that results in increased expression of IL-6.

Absence of Dectin-1 Ligands Prevent Th17 Cell Induction by Dermal DCs

To examine the function of dermal DC subsets in the induction of Th17 cell responses, we first compared expression of Dectin-1 by each subset. Dectin-1 is expressed by LCs and CD11b+ dDCs but not by CD103+ dDCs (Figure 5A). The absence of Dectin-1 on CD103+ dDCs is consistent with our previous observation that this DC subset was required for Th1 but not Th17 cell induction (Igyártó et al., 2011).

Because Th17 cell differentiation is reduced but not ablated in the absence of LCs and because CD11b+ DCs in the lung are required for Th17 cell responses during Aspergillus fumigatus infection (Schlitzer et al., 2013), we hypothesized that Dectin-1 on CD103+ dDCs is required for Th17 cell differentiation. Macrophage galactose C-type lectin 2 (Mgl2) is expressed by most CD11b+ dDCs. Administration of diphtheria toxin (DT) to the recently reported Mgl2-DTR mice results in ablation of CD11b+ dDCs and LCs (Figures S6A–S6C; Kumamoto et al., 2013). DT-treated Mgl2-DTR mice have reduced Th17 cell differentiation that could result from deletion of either subset (Figure S6D). To selectively ablate CD11b+ dDCs, we generated Mgl2-DTR/WT chimeric mice (Figure S6C). Notably, antigen-specific T cell expansion and Th cell phenotype was unaltered in DT-treated Mgl2-DTR→WT mice infected with Enol1-Ag, indicating that CD11b+ DDCs are not required for Th17 cell differentiation (Figures S6B and S5C). To determine whether this DC subset could be sufficient for Th17 cell induction, we generated LC−×Batf3−/−mice that have a genetic absence of both LCs and CD103+ dDCs. Enol1-Ag infection of these mice in which CD11b+ dDCs are the dominant skin-resident DC subset demonstrated defective Th1 and Th17 cell differentiation (Figures 5D and 5E) that is consistent with the absence of Th17 cells generated by LCs and Th1 cells induced by CD103+ dDCs. Thus, CD11b+ dDCs are neither necessary nor sufficient for Th17 cell generation during epicutaneous infection with C. albicans.

Despite the equivalent degree of T cells expansion in LC−×Batf3−/−mice, we considered the possibility that CD11b+ dDCs have limited access to antigen during an epicutaneous infection. In wild-type mice, we compared epicutaneous with dermal infection by using the same inoculum of Enol1-Ag. Though T cell expansion and Th1 cell differentiation was similar, Th17 cell differentiation was greatly reduced in response to intradermal infection (Figures 6A and 6B). Induction of Th17 cell was also absent in response to an i.v. systemic infection (Figures S7A and S7B). Notably, addition of the Dectin-1 ligand, curdlan, with the intradermal inoculation rescued the Th17 cell response, suggesting that Dectin-1 ligation does not occur in the dermis during C. albicans infection but that CD11b+ dDCs do have the capacity to induce Th17 cells (Figure 6C). During epicutaneous C. albicans infection, yeast are found on the epidermis and invasive filamentous forms in the dermis (Figure 6D; Gow et al., 2012). To test
whether hyphal forms of *C. albicans* are unable to induce Th17 cells due to the absence of Dectin-1 ligands, we infected mice epicutaneously with *Hwp1*-Ag with the addition of exogenous curdian or vehicle (Figure 6E). The addition of Dectin-1 ligands rescued the ability of antigen expressed by hyphal forms of *C. albicans* to generate Th17 cell responses.

**Compartmentalization of Th Cell Responses**

The ability of *C. albicans* to induce Th17 cells in response to an epidermal infection and Th1 cells in response to an invasive infection raises the possibility that specific Th cell phenotypes could provide protection at distinct anatomical sites. To test this hypothesis, we epicutaneously infected WT, *Il6*^-/-^, and *Batf3*^-/-^ mice with *Eno1*-Ag. As expected, *Il6*^-/-^ mice generated a robust Th1 cell response in the absence of Th17 cells and *Batf3*^-/-^ mice developed a strong Th17 cell response in the absence of Th1 cells (Figure 7A). Separate cohorts of mice were re-challenged by skin infection 9 days after spontaneous clearance of the primary infection. The number of colony forming units (CFUs) found in naive mice challenged with SC5314 was similar in all three groups (open symbols) (Figure 7B). Notably, though IL-6 is an innate cytokine important for resistance to systemic *C. albicans* infection, naive *Il6*^-/-^ mice did not develop an exaggerated cutaneous infection (van Enckevort et al., 1999). Secondly infected (closed symbols) WT mice had an approximate 10-fold increase in fungal resistance. In contrast, *Batf3*^-/-^ mice had approximately 100-fold increase in pathogen resistance and *Il6*^-/-^ mice showed no increased resistance to re-infection compared to naive mice (Figure 7B). To test whether this was a CD4^+^ T-cell-dependent process, we purified CD4^+^ T cells from *Il6*^-/-^ and *Batf3*^-/-^ mice infected 7 days earlier and adoptively transferred them into naive hosts that were then skin infected. Naive recipients of CD4^+^ T cells from *Batf3*^-/-^ but not *Il6*^-/-^ mice were protected against a cutaneous infection. Thus, the Th17 cells that arise in response to a cutaneous *C. albicans* infection provide protection against subsequent skin challenges (Figure 7C).

In contrast, systemic challenge of previously skin-infected mice by i.v. inoculation revealed protection in WT and *Il6*^-/-^ mice but not in *Batf3*^-/-^ mice (Figure 7D). Similarly, adoptive transfer of CD4^+^ T cells from skin-infected *Il6*^-/-^ mice but not *Batf3*^-/-^ mice into naive recipients conferred protection against systemic candidiasis (Figure 7E). Thus, mice with expanded numbers of Th17 cell effectors were more resistant to cutaneous *C. albicans* challenge whereas mice with expanded numbers of Th1 cells were more resistant to systemic challenge. We conclude that Th cell effector cells appear to be functionally compartmentalized, with Th17 cells providing skin protection and Th1 cells providing systemic protection.

**DISCUSSION**

Herein we have demonstrated that the differentiation of Th17 cells in response to *C. albicans* skin infection occurs during infection with *C. albicans* yeast but not pseudo-hyphae. Th17 cell induction required the engagement of Dectin-1 and subsequent expression of IL-6 by Langerhans cells. CD11b^+^ dDCs expressed Dectin-1 but were neither necessary nor sufficient for Th17 cell responses. The inability of *C. albicans* in the dermis to induce Th17 cells resulted from a morphologic change from yeast to pseudo-hyphae that occurred during invasion and could be rescued by the addition of exogenous Dectin-1 ligand. Finally, we found that Th17 cell expansion provided protection against cutaneous but not systemic challenge whereas the Th1 cell expansion provided protection against systemic but not cutaneous challenge.

The ability of *C. albicans* morphology to dictate immune responses has been an area of considerable controversy. Our
Dermal Infection and *C. albicans* Pseudo-Hyphae Do Not Induce Th17 Cells without Exogenous Dectin-1 Ligands

(A and B) WT mice were infected with an equivalent inoculum of *Eno1-Ag* *C. albicans* on the epidermis (white) or by intradermal injection (black). Expansion (A) and cytokine production (B) by PMA-and-ionomycin-stimulated TEs cells isolated from skin draining lymph nodes is shown. (C) As in (A), WT mice were infected epicutaneously or by dermal injection into which purified curdlan or vehicle has been added to the inoculum. CSFE dilution and expression of IL-17A by PMA-and-ionomycin-stimulated TEs cells is shown. (D) PAS stain of skin 2 days after *C. albicans* infection showing budding yeast (asterisk) restricted to the epidermis and numerous penetrating pseudo-hyphae in the dermis (arrowhead) (scale bar represents 150 μM). (E) The expression of IL-17A and IFN-γ by PMA-and-ionomycin-stimulated TEs cells 4 days after epicutaneous infection of WT mice with *Eno1-Ag* or *Hwp1-Ag* is shown. Curdlan or vehicle alone was provided by dermal injection at the time of infection. "Neg" represents mock infected WT mice. Scales represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. Data are representative of at least three independent experiments. See also Figure S7.

**Figure 6.**

**Data using mutants that are locked into yeast (chp1/cph1) or pseudo-hyphae (tup1/tup1) as well as a recombinant *C. albicans* strain in which model antigens are expressed exclusively by pseudo-hyphae (*Hwp1-Ag*) demonstrate that, in vivo, *C. albicans* yeast but not pseudo-hyphae induce Th17 cell differentiation. This is consistent with the finding that the ligand for Dectin-1 is largely accessible in the scars or patches of budding yeasts (Cabib and Bowers, 1971; Gantner et al., 2005; Gow et al., 2012). Using in vitro methods, others have found that *C. albicans* pseudo-hyphae can induce Dectin-1-mediated Th17 cell differentiation through an IL-1β-dependent mechanism as well as induce Th2 or Tr1 cell responses (Cheng et al., 2011; d’Ostiani et al., 2000). These data can be reconciled by the differing availability in vitro versus in vivo of Dectin-1 ligands on morphologic forms of *C. albicans* (Marakalala et al., 2013).

Differentiation of Th17 cells requires TGF-β as well as IL-1β and/or IL-6 depending on the site of immunization (Hu et al., 2011). Using total cytokine-deficient mice, we found that Th17 cell development during skin infection is dependent on both IL-1β and IL-6. By generating mice with LC-selective defects of TGF-β, IL-1β, and IL-6, we found that LCs were a non-redundant source of only IL-6. Though LC-derived IL-1β and TGF-β were not required, there are many other cellular sources of these cytokines that could either be the primary source during infection or provide functionally sufficient quantities in the absence of LCs. Notably, specific ablation of MyD88 in LCs resulted in reduced expression of IL-6 and defective Th17 cell differentiation during *C. albicans* infection, suggesting that IL-1β could potentiate IL-6 expression by LCs (Haley et al., 2012). It is important to note that Dectin-1 also cooperates with toll-like receptor-2 (TLR-2) and TLR-4 to mount pro-inflammatory responses (Ferwerda et al., 2008; Gantner et al., 2003). In addition to any possible role for IL-1β, we found that engagement of Dectin-1 on LCs was required for optimal IL-6 expression. Infection with a Dectin-1-independent strain induced only low expression of IL-6 by LCs that failed to induce Th17 cell differentiation. Addition of exogenous IL-6 was able to rescue the Th17 cell phenotype. Similarly, patients with Dectin-1 deficiency suffer from CMC and have defective IL-6 production in response to *C. albicans* (Ferwerda et al., 2009). Thus, we have defined a model in which interaction of LCs with *C. albicans* yeast results in a Dectin-1-dependent expression of IL-6 that is required for Th17 cell differentiation during skin infection.

In the dermis, Dectin-1 is expressed by CD11b⁺ dDCs but not CD103⁺ dDCs. The absence of Dectin-1 on CD103⁺ dDCs is consistent with our prior observations that these cells were required for induction of Th1 cell differentiation (Iguyáró et al., 2011). Despite expressing Dectin-1, CD11b⁺ dDCs were neither necessary nor sufficient for optimal induction of Th17 cell responses to either an epicutaneous or dermal infection with *C. albicans*. Importantly, during a dermal infection or an epicutaneous infection with *Hwp1-Ag*, situations in which antigen bypasses LCs in the epidermis, efficient Th17 cell differentiation was restored by addition of the exogenous Dectin-1 ligand, curdlan. CD11b⁺ dDCs have been reported to drive Th2 cell responses to papain immunization and nippostrongylus infection.
Figure 7. Th1 and Th17 Cells Provide Compartmentalized Protection to C. albicans

WT, Il6<sup>−/−</sup>, and Batf3<sup>−/−</sup> mice were epicutaneously infected with Eno1-Ag. (A) The expression of IL-17A and IFN-γ by PMA-and-ionomycin-stimulated CD4<sup>+</sup> TE<sub>x</sub> cells isolated from skin draining lymph nodes 4 days after infection is shown.

(B) The indicated strains of mice were mock infected (white) or skin infected with SC5314. Mice were re-challenged in the skin with SC5314 9 days after shown.

(C) CD4<sup>+</sup> T cells were purified from skin draining LN and spleen of naive WT, SC5314-infected Il6<sup>−/−</sup>, or SC5314-infected Batf3<sup>−/−</sup> mice 7 days after infection and adoptively transferred into naive WT recipients. Recipients were skin infected with SC5314 1 day after transfer and CFU was assessed at the infected area 2 days later.

(D) As in (B) except that mice were challenged by i.v. infection with 10<sup>5</sup> SC5314. Scales represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

(E) As in (C) except that mice were challenged by i.v. infection with 10<sup>7</sup> SC5314. Fungal burdens in the kidneys expressed as CFU/g is shown.

(Gao et al., 2013; Kumamoto et al., 2013). Our data suggest that in addition to Th2 cells, these cells might also have the capacity to induce Th17 cell differentiation given the appropriate adjuvant. Indeed, similar IRF4<sup>+</sup>CD11b<sup>+</sup> DCs can produce Th17 cell responses in other tissues (Persson et al., 2013; Schlitzer et al., 2013). Thus, the inability of CD11b<sup>+</sup> dDCs to induce Th17 cells is not intrinsic to CD11b<sup>+</sup> dDCs but results from the morphologic change of C. albicans into pseudo-hyphae as it invades into the dermis and probably represents an adaptation of C. albicans to evade immune responses.

Humans are chronically exposed to C. albicans and circulating anti-Candida CD4<sup>+</sup> Th1 and Th17 cells have been identified (Zielinski et al., 2012). The importance of adaptive responses to C. albicans infection has been demonstrated by the high prevalence of mucocutaneous candidiasis in patients with Th17 cell defects (e.g., hyper IgE syndrome) and some immunodeficiencies (e.g., HIV) (McDonald, 2012). Our results as well as the work of others has found that adaptive responses to C. albicans infection in mice are also dominated by Th1 and Th17 cells and provide protection from secondary infection, thus mimicking the response seen in humans (Conti and Gaffen, 2010; Eyerich et al., 2008; Puel et al., 2011; Zielinski et al., 2012). We found that Batf3<sup>−/−</sup> mice lacking CD103<sup>+</sup> dDCs and Il6<sup>−/−</sup> mice developed either greatly exaggerated or absent Th17 cell development, respectively, without any alteration in skin innate responses to C. albicans. Secondary skin infection of mice with exaggerated Th17 cell expansion resulted in significant increase in protection compared with mice lacking Th17 cells that showed no evidence of protection. Mice receiving exogenous C. albicans-primed Th17 but not Th1 cells also were protected from skin infection. Thus, consistent with the well-documented role of Th17 cells in protection from CMC, Th17 cell expansion provided protection from secondary skin infection (Eyerich et al., 2008; McDonald, 2012). In addition, the ability of C. albicans yeast to drive Th17 cell responses and provide protection argues against the concept that the failure of yeast to generate Th17 cell is a mechanism that maintains C. albicans commensalism (Bonifazi et al., 2009; Romani, 2011).

Notably, we found that Th17 cell expansion failed to provide protection from systemic infection. Rather, enhanced protection from systemic challenge occurred in Il6<sup>−/−</sup> mice that developed exaggerated Th1 cells as well as naive recipients of primed CD4<sup>+</sup> T cells isolated from infected Il6<sup>−/−</sup> mice. Notably, this protection developed despite an enhanced susceptibility to primary disseminated C. albicans that occurs in Il6<sup>−/−</sup> mice (van Enckevort et al., 1999). It was also independent of the phenomenon of reprogrammed monocytes that can provide protection from systemic infection through an IL-6-dependent mechanism (Quintin et al., 2012). Notably, patients with mutations in STAT1 that reduce signaling of IFN-γ develop increased rates of systemic infection with C. albicans and other fungi (Puel et al., 2011; Sampaio et al., 2013). We speculate that IFN-γ or granulocyte-macrophage colony-stimulating factor (GM-CSF) derived from Th1 cell effectors might enhance the anti-Candida responses by promoting increased NK and/or macrophage activity (Bär et al., 2014).

In summary, these data reveal a role for C. albicans morphology and interaction with a single DC subset for the generation of Th17 cell responses. In addition, the tissue-selective...
protection afforded by Th17 and Th1 cell effectors reveals a
correlation of Th cell function that suggests a
rational basis for the development of Th-cell-phenotype-specific
C. albicans vaccines.

EXPERIMENTAL PROCEDURES

Mice
Mgl2-GFP-DTR/Rv (referred to as Mgl2-DTR), human Langerin-DTA (LC−), human
Langerin-DTA × Batf3−/− (referred to as LC− × Batf3−/−), huLangerin-DTR,
uLuangerin-eGFP, human Langerin-creER(T2), YFP, Tgb1T, Tgb2T, and
Ciec7a−/− mice have been previously described (Bohr et al., 2012; Kaplan et al., 2005; Kissenyfenni et al., 2005; Kumamoto et al., 2013; Taylor et al., 2007; Welly et al., 2013). B6;129S7-Itgb2−/− and B6;129S7-Itgb2−/− were purchased from The
Jackson Laboratory. C57BL/6 and B6-Ly5.2/Cr were purchased from the
National Cancer Institute or Charles River laboratories. CD90.1 Rag1−/−, CD4−
TEα TCR-transgenic mice that recognize E364-366 in I-Aα on C57BL/6 back
grounds were also used (Grubin et al., 1997). All experiments were performed
with 6- to 12-week-old and sex-matched mice. Mice were housed in microiso
lator cages and fed irradiated food and acidified water. The University of
Minnesota institutional care and use committee approved all mouse protocols.

Generation and Testing of Bone Marrow Chimeric Mice
6-week-old cytokine-deficient, wild-type C57BL/6 or Ly5.2 congenically
marked mice were irradiated with X-ray irradiator as described previously.
The mice received two split doses at 500 cGy each. The following day, 5 × 106 bone marrow cells isolated from specified mice were injected intrave
rously. Mice were rested for at least 6 weeks prior to experiments. The effi
 ciency of chimerism was determined by flow cytometry of congenic markers
on PBMC, lymph node, and epidermis.

DC Depletion with Diphtheria Toxin
Mgl2-DTR and huLangerin-DTR mice were i.p. injected with 1 mg of diphtheria
Toxin (List Biological Laboratories) 2 days before infection, as previously
described (Bohr et al., 2010; Igantaré et al., 2011).

Tamoxifen Treatment
Tamoxifen (Sigma Aldrich) was dissolved in corn oil (Sigma Aldrich) and 10%
ethanol and was administered 5 consecutive days by i.p. injection at 0.05 mg/g
of mouse weight.

Antibodies
Fluoroochrome-conjugated antibodies to CD4 (GK1.5), CD11b (M1/70), CD11c
(N418), CD45.1 (A20), CD45.2 (104), CD90.1 (OX-7), CD103 (2E7), I-A/I-E
(M5/114.15.2), Dectin-1 (RH1), IFN-γ (XM1G2.1), IL-10 (JES5-16E3), and
IL-17A (TC11-18H10.1) were purchased from BioLegend. Anti-mouse Langer
in (L31), IL-17F (18F10), IL-22 (1H5PSWR), and Foxp3 (FJK-16B) were acquired from ebioscience.

Adaptive T Cell Transfer
T cells were adaptively transferred as previously described (Igantaré et al.,
2011). In brief, skin draining lymph nodes, spleen, and mesenteric lymph
node of TEs mice were disrupted through a cell strainer, washed with sterile
HBSS, and labeled with CFSE (Invitrogen) in accordance with the manufac
urer’s instructions. The cells after two washes were resuspended in sterile PBS at a concentration of 1 × 106 cell/ml and 300 µl (3 × 106 cells) were
injected intravenously into recipients.

In certain experiments, single-cell suspension from secondary lymphoid
organs from either naive wild-type or skin-infected Il6− or Batf5-deficient
mice were obtained. CD4+ T cells were magnetically isolated at ≥95% purity
(STEMCELL Technologies) according to manufacturer’s directions. A total of
5 × 106 million purified CD4+ T cells were transferred into naive hosts in
300 µl PBS.

Flow Cytometry
Single-cell suspensions of epidermal cells were obtained as previously defined
(Kaplan et al., 2005). In brief, skin from ear or trunk were obtained and
incubated for 2 hr at 37°C in 0.3% trypsin (Sigma-Aldrich) in 150 mM NaCl,
0.5 mM KCl, 0.5 mM glucose. The epidermis was physically separated from
the dermis. Dermal cells were obtained from whole flank skin. Samples were
minced and incubated for 2 hr at 37°C in collagenase XI (4,830 U/ml, Sigma),
hyaluronidase (260 U/ml, Sigma), DNase (50 ml/ml, ICN), 10 mM HEPES
(Sigma) in RPMI (Invitrogen). The resulting cells were filtered through a
40 μm filter. Lymph node (axillary, brachial, and inguinal) and spleen cells
were incubated in 400 and 150 U/ml Collagenase D for preparation of dendritic
cells or smashed through a filter for lymphocytes (Roche Applied Science),
respectively, for 90 min prior to erythroid cell lysis in ACK buffer (BioWhittaker).

Single-cell suspensions were pretreated for 10 min at 4°C with 2.4G2 except
when anti-Rat secondaries were used, in which case cells were blocked
with mouse Ig (Sigma). For evaluating cytokine expression, cells were incu
bated for 5 hr in complete IDEMEM supplemented with PMA (50 ng/ml) and
ionomycin (1.5 µM; Sigma-Aldrich), with GolgiStop (BD PharMingen) added
for the final 4 hr. The intracellular cytokine staining was performed with BD
Bioscience Cytofix/Cytoperm kit (BD Biosciences) in accordance with the
manufacturer’s instructions. Samples were analyzed on LSRII flow cytomets
(TD Biosciences). Data were analyzed with FlowJo software (TreeStar).
Detection of 2W1S-specific CD4+ T cells was performed as published with an
I-Ab-2W1S tetramer (a kind gift of M. Jenkins) after ex vivo stimulation of
secondary organs as previously described (Moon et al., 2007).

DC Sorting by Flow Cytometry and qPCR
Single-cell suspensions of LN cells were enriched by CD11c MACS positive
positively sorted as described (Milenyi Biotech). LCs were sorted with a FACSAria cell sorter based as GFP+, MHCI+, CD11c+, CD11b+, CD103 CD68−. RNA was isolated with an
RTeasy Mini Kit (Qiagen) and quantified from Nanodrop readings (Nanodrop).
cDNA was generated with a High Capacity cDNA Reverse Transcription
Kit (Applied Biosystems). TaqMan Gene Expression Master Mix and TaqMan Gene Expression Assays for IL-1, IL-6, and IL-12p40 was used. ABI
Prism 7900HT (Applied Biosystems) were used to complete the qPCR.
All kits were completed according the manufacturer’s instructions. All Ct
values were normalized to HPRT expression and are shown as 2−ΔΔCt to naive.

Candida albicans Strains
Recombinant C. albicans used in this study was derived from SC5314 and was
designed as previously described (Fonzi and Irwin, 1993; Igantaré et al., 2011).
They were grown in rich medium at 30°C (YPAD) or in synthetic complete me-
dium. Escherichia coli strain XLI-blue (Strategene), growth conditions, DNA
manipulations, and primer design and synthesis were essentially as described
previously. Transformants were selected on YPAD medium containing
400 µg/ml nourseothricin and were screened by PCR with oligonucleotide
primers listed (Figure S2C). In brief, strain Hwp1−/−eGFP-Ag was constructed
as follows: plasmid pMG2268 was PCR with Primers S75 and S266. PCR
product was transformed into wild-type strain SC5314 by standard methods
(Igantaré et al., 2011). Transformants were selected on YPAD medium containing
400 µg/ml nourseothricin. Integration at the Hwp1 locus was confirmed with two primers (877/3290) confirming integration of GFP-antigen at Hwp1P.
C. albicans strains x220175.016, yeast locked strain (JKC19), and filament
locked strain (BCa2-9) were kindly provided by Dr. Neal Gow (University of
Aberdeen) (Braun and Johnson, 1997; Liu et al., 1994; MacCallum, 2012; Marakalala et al., 2013). Growth of fungi occurred after inoculation of a colony
at 30°C in YPAD overnight and, the next day, diluted 1:10 and cultured either at
30°C in YPAD until OD600 reached 1.5 for yeast morphology or at 37°C in RPMI
with 10% PBS for 3 hr for filamentous forms.

Infection Models
The skin infection was performed as described (Igantaré et al., 2011). In brief,
mice were first anesthetized with a mixture of ketamine and xylazine (100/10
mg/kg body weight), shaved on the back with electric clipper, and chemi
cally depliated with Nair hair remover (Church & Dwight) per the manufac
urer’s instructions. The stratum corneum was removed with 15 strokes with
220 grit sandpaper (3M). After washing with sterile PBS, 2 × 106 C. albicans
in 50 µl of sterile PBS was applied to the skin. In some experiments, same
dose of C. albicans or 50 µg curdian was injected intradermally spaced over
10 sites on the back. Where indicated, recombinant murine IL-6 (BioLegend)
was injected into 4 different sites in the dorsum of the mice intradermally at

a total dose of 1 µg per mice. i.v. infections with En01-Ag and Hwp1-Ag for assessment of T cell expansion were performed at 3 × 10^5 CFU in PBS.

In re-challenge experiments, previously mock or epicutaneously infected mice were challenged with 1 × 10^6 CFU C. albicans by being intradermally injected into a single site or by 1 × 10^6 CFU intravenously. Skin and kidneys were harvested from mice 3 days later and homogenized and serially diluted onto YPAD plates and incubated at 30°C for 24–48 hr to assess the number of colony-forming units.

**LC Targeting with Anti-huLangerin**

Anti-human-Langerin antibodies (2G3) conjugated to antigen were generated and administered as previously described (Gyártó et al., 2011). In brief, WT and huLangerin-DTR mice were immunized by intraperitoneal injection of 1.0 µg of 2G3-Ex in 100 µl of sterile PBS. Six hours later, the mice were infected with the pathogen strains noted as above.

**Histology**

Immunofluorescence on ear epidermis was performed as previously described (Kaplan et al., 2009). Images were captured with a microscope (DM5500; Leica) with digital system and LAS AF software (v.1.5.1). Periodic Acid-Schiff (PAS) staining for fungal invasion was performed 2 days after infection with SC5314 on formaldehyde-fixed frozen sections with commercial PAS staining kit (Sigma-Aldrich).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.01.008.

**AUTHOR CONTRIBUTIONS**

S.W.K., B.Z.I., and D.H.K. designed experiments. S.W.K and B.Z.I. performed experiments. M.-G.-N. and J.B. helped in construction and validation of C. albicans strain En01-Ag and Hwp1-Ag. A.I. and G.D.B. assisted with the development of experimental models. Y.K. and A.I. developed and provided Mgl2-DTR mice. E.J., J.A.M., and R.A.D. assisted with experiments. S.M.Z. and G.Z. developed and provided monoclonal antibody 2G3 against human Langerin. S.W.K., B.Z.I., and D.H.K. wrote the manuscript. All authors participated in discussions of experimental results and edited the manuscript.

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