

Skin dendritic cells induce follicular helper T cells and protective humoral immune responses

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Background: The contribution of individual subsets of dendritic cells (DCs) to generation of adaptive immunity is central to understanding immune homeostasis and protective immune responses.

Objective: We sought to define functions for steady-state skin DCs.

Methods: We present an approach in which we restrict antigen presentation to individual DC subsets in the skin and monitor the effects on endogenous antigen-specific CD4⁺ T- and B-cell responses.

Results: Presentation of foreign antigen by Langerhans cells (LC) in the absence of exogenous adjuvant led to a large expansion of T follicular helper (T_{FH}) cells. This was accompanied by B-cell activation, germinal center formation, and protective antibody responses against influenza. The expansion of T_{FH} cells and antibody responses could be elicited by both systemic and topical skin immunization. T_{FH} cell induction was not restricted to LCs and occurred in response to antigen presentation by CD103⁺ dermal DCs. CD103⁺ DCs, despite inducing similar T_{FH} responses as LCs, were less efficient in induction of germinal center B cells and humoral immune responses. We also found that skin DCs are sufficient to expand CXCR5⁺ T_{FH} cells through an IL-6- and IFN- α/β receptor-independent mechanism, but B cells were required for sustained Bcl-6⁺ expression.

Conclusions: These data demonstrate that a major unappreciated function of skin DCs is their promotion of T_{FH} cells and humoral immune responses that potentially represent

an efficient approach for vaccination. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Steady-state conditions, skin dendritic cells, T follicular helper cells, protective humoral immune responses

The dendritic cell (DC) paradigm states that after a pathogen encounter, DCs become activated and migrate to the secondary lymphoid organs, where they initiate expansion and differentiation of pathogen-specific T-cell responses.¹ In the steady state DCs at barrier sites acquire self-antigens and benign environmental antigens. The presentation of these antigens by immature DCs results in T-cell tolerance through either activation-induced cell death or induction of anergic/regulatory T (Treg) cells.² This is believed to be the mechanism for the maintenance of peripheral tolerance and has been hypothesized to be a targetable pathway to induce immune tolerance for the treatment of autoimmune diseases.

In mouse skin there are 2 distinct populations of DCs that express the C-type lectin langerin: Langerhans cells (LCs), which reside in the epidermis, and CD103⁺ DCs, which reside in the dermis.¹ We have previously demonstrated that LCs and CD103⁺ DCs induced opposing T_H responses against *Candida albicans*. As such, LCs were necessary and sufficient for *in vivo* induction of T_H17 responses, whereas CD103⁺ DCs were required for cross-presentation to CD8 T cells and T_H1 responses.³ The role of CD103⁺ DCs in cross-presentation has been supported by other studies using different models and also antigen targeting.³⁻⁶ In the setting of contact hypersensitivity, the function of LCs and CD103⁺ cells remains controversial.⁷ 2,4-Dinitrochlorobenzene-induced tolerance was dependent on LC-induced Treg cell expansion.⁸ In addition, LCs have been reported to promote deletion of antigen-specific CD4⁺ T cells after complete Freund adjuvant peptide immunization⁹ and expansion of Treg cells during *Leishmania* species infection.¹⁰ LCs are also required for the induction of protective antibody responses after epicutaneous patch immunization.¹¹

The function of langerin-expressing cells in the steady state can be examined by using intraperitoneal injection of low amounts of anti-langerin mAb/antigen conjugates. Because ligation of langerin does not activate LCs and CD103⁺ DCs, this technique assays the effect of antigen presentation of langerin-positive DCs in the absence of exogenous adjuvants. Anti-mouse langerin/myelin oligodendrocyte glycoprotein (MOG) conjugates induced expansion of antigen-specific transgenic Treg cells and provided subsequent protection from experimental autoimmune encephalomyelitis (EAE).¹² This finding suggests that langerin-expressing DCs (LCs and CD103⁺ DCs) promote tolerance through Treg cell expansion and is consistent with earlier studies using the DEC-205 mAb to target antigen to other DC subsets under homeostatic conditions.¹³

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Abbreviations used

CFA:	Complete Freund adjuvant
CFSE:	Carboxyfluorescein succinimidyl ester
DC:	Dendritic cell
EAE:	Experimental autoimmune encephalomyelitis
FluHA1:	Influenza hemagglutinin A1
FoxP3:	Forkhead box protein 3
GC:	Germinal center
HRP:	Horse radish peroxidase
IFNAR:	IFN- α/β receptor
LC:	Langerhans cells
LN:	Lymph node
MHCII:	MHC class II
MOG:	Myelin oligodendrocyte glycoprotein
PD-1:	Programmed cell death 1
PR8:	Puerto Rico 8
ROR γ t:	Retinoic acid–related orphan receptor γ t
T-bet:	T-box transcription factor
TCR:	T-cell receptor
T _{FH} :	T follicular helper
Treg:	Regulatory T

The contribution of individual subsets of DCs to the generation of adaptive immunity is central to understanding immune homeostasis and protective immune responses. To date, DC function has been studied either *in vitro* or by using adoptive transfer of T-cell receptor (TCR) transgenic T cells. To determine the functional consequence of foreign antigen presentation without adjuvants exclusively by LCs or CD103⁺ DCs, we developed an approach in which we restrict antigen presentation to these individual DC subsets and monitor the effects on endogenous antigen-specific CD4⁺ T-cell responses by using MHC class II (MHCII) tetramers.¹⁴ We also developed a novel system for concomitant analysis of endogenous B-cell responses. Using these techniques, we defined new functions for LCs and CD103⁺ DCs in T follicular helper (T_{FH}) cell induction and humoral immune responses.

METHODS**Mice**

huLangerin,¹⁵ huLangerin-Cre-I-AB¹⁶, and Batf3^{-/-}¹⁷ mice have been previously described. CD90.1 congenic TE α Rag1^{-/-} Cd4 TCR transgenic mice to I-E α ₅₂₋₆₈ on the C57BL/6 background¹⁸ were obtained from M. Jenkins (University of Minnesota), μ MT and CD11c-Cre-MHCII mice were from K. Hogquist (University of Minnesota), and IFN- α/β receptor (IFNAR)^{-/-} mice were from M. Mescher (University of Minnesota). IL-6^{-/-} mice on the C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, Me). All experiments were performed with 6- to 12-week-old female mice. Mice were housed in microisolator cages and fed irradiated food and acidified water. The University of Minnesota Institutional Care and Use Committee approved all mouse protocols.

Antibodies and reagents

Fluorescence-conjugated antibodies to CD4, CD11b, CD11c, CD40, B220, CD44, CD86, CD90.1, CD90.2, CD103, Gr-1, F4/80, and I-A/I-E were purchased from BioLegend (San Diego, Calif). Antibodies to forkhead box protein 3 (FoxP3), T-box transcription factor (T-bet), programmed cell death 1 (PD-1), CXCR5, Bcl-6, Gata-3, retinoic acid–related orphan receptor γ t (ROR γ t), and Live/Dead dye were acquired from eBioscience (San Diego, Calif). Anti-langerin (929F3) was from Dendritics (Lyon, France). Anti-human/anti-mouse langerin mAb and conjugates (2G3-E α , 2G3-2W1S,

4C7-2W1S, and 2G3–influenza hemagglutinin A1 [FluHA1]) were generated in house, as previously described.^{3,19} E α (I-E α ₅₂₋₆₈) is a well-characterized immunodominant T-cell epitope from the I-E α MHCII molecule recognized by transgenic TE α cells in the context of I-Ab.¹⁸ 2W1S is a variant of peptides 52 to 68 from the I-E α chain.¹⁴ 2W1S binds to the I-Ab MHCII molecule expressed in C57BL/6 (B6) mice and is immunogenic in this strain. FluHA-1 represents influenza hemagglutinin A1 (influenza A virus [A/Puerto Rico/8/34{H1N1}]) residues 18 to 331.¹⁹

Adoptive T-cell transfer

T cells were adoptively transferred, as previously described.³ Briefly, skin-draining lymph nodes (LNs), spleens, and mesenteric LNs of TE α TCR transgenic mice were disrupted through a cell strainer, washed with sterile HBSS, and labeled with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions. The cells were resuspended in sterile PBS at a concentration of 1×10^6 cells/mL, and 300 μ L (3×10^5 cells) was injected intravenously into different mouse strains.

LC and CD103⁺ DC targeting with anti-langerin antibody by means of intraperitoneal injection

Cohorts of mice were injected with either 10 μ g of 2G3-AF647 or 10 μ g of 4C7-AF647. Mice were killed 16 hours later, and the presence of the AF647 signal was analyzed by means of flow cytometry in cells isolated from the epidermis, LNs, and spleen, as previously described.³ In separate experiments cohorts of mice were adoptively transferred with 3×10^5 TE α cells. Twenty-four hours later, mice were immunized by means of intraperitoneal injection of 1 μ g of 2G3-E α , and skin-draining LNs were harvested at day 4. Cohorts of mice were injected with either 1 μ g of 2G3-2W1S or 1 μ g of 4C7-2W1S to define endogenous T-cell responses. Six skin-draining LNs and spleens were harvested at the indicated time points and analyzed by using flow cytometry.

Topical application of antigen

Mouse back skin was wet-shaved under anesthesia by using razorblades. One hundred micrograms of 2W1S or MOG₃₅₋₅₅ (GenScript, Piscataway, NJ) diluted in 100 μ L of endotoxin-free PBS was smeared onto the skin. A separate cohort of mice was painted with 1 μ g of 2G3-2W1S.

Flow cytometry

Single-cell suspensions were obtained and stained, as previously described.³ All the flow cytometric plots presented in this article were pregated on live (using Live/Dead stain) and singlet events. Intracellular transcription factor staining was performed with the BD Bioscience Cytofix/Cytoperm kit (BD Biosciences, San Jose, Calif), according to the manufacturer's instructions. Samples were analyzed on LSR II flow cytometers (BD Biosciences). Data were analyzed with FlowJo software (TreeStar, Ashland, Ore). Boolean gating was used to identify single transcription factor–expressing cells, and data are displayed as percentages of total values in a pie graph.

2W1S tetramer enrichment

Single-cell suspensions of LNs and spleens were stained for 1 hour at room temperature with 2W1S:I-A^b–streptavidin–APC tetramers (a generous gift of M. Jenkins). Samples were then enriched for bead-bound cells on magnetized columns, as previously described.¹⁴

Endogenous B-cell responses

Single-cell suspension of LNs from MD4, naive, and 2G3-immunized WT and huLangerin-positive mice were incubated *ex vivo* with AF647-conjugated 2G3 on ice for 30 minutes. After washing, the cells were stained for Live/Dead, CD11c, CD90.2, CD45.2, Gr-1, MHCII, B220, GL-7, and CD38 markers and analyzed on a flow cytometer.

Assessment of humoral responses

WT and huLangerin mice were injected intraperitoneally with 1 μ g of 2G3 at days 0 and day 14. $LC^{-/-}$ mice were treated in a similar way but with 4C7. Serum samples were obtained before and 21 days after immunization by using BD Microtainer SST tubes (BD, Franklin Lakes, NJ) and stored at -80°C . ELISA plates were coated with 0.25 μ g of hIgG₄ protein to determine antigen-specific antibody titers. Serial dilutions of serum in blocking buffer (TBS; Pierce, Rockford, Ill) were incubated in wells overnight at 4°C . After washing, plates were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, Pa) in blocking solution for 2 hours at 37°C , washed and developed with HRP substrate, and read at 405 nm. Antibody titer data are plotted on log scales. Assays for isotyping antigen-specific antibodies in the serum used biotinylated goat anti-mouse IgG₁, IgG_{2b}, IgG₃, and IgA polyclonal reagents and biotinylated anti-mouse IgE monoclonal 2G3 (SouthernBiotech, Birmingham, Ala) as detecting reagents, followed by development with NeutrAvidin HRP (Pierce, Rockford, Ill).

Intranasal influenza infection

WT and huLangerin mice were injected intraperitoneally with 1 μ g of 2G3-FluHA1 at days 0 and 14. On day 21, the mice were infected intranasally with 2×10^4 plaque-forming units of Puerto Rico 8 (PR8) influenza virus. Body weights and survival curves were obtained. Mice that lost 25% of their original weight were killed.

Quantitative RT-PCR for viral load

Lung samples were collected 9 days after influenza infection, and viral load was assessed by using a standard quantitative RT-PCR protocol. Briefly, total RNA from lung samples was extracted with the RNeasy Mini Kit (Qiagen, Valencia, Calif), according to the manufacturer's protocol. Reverse transcription was performed with the Superscript III kit (Invitrogen, Carlsbad, Calif), and the RT-PCR reaction was performed with SYBR-green (Applied Biosystems, Grand Island, NY). For amplification, PR8 NP-specific primers (ACGGCTGGTCTGACTCACAT and TCCATTCCGGTGCGAACAAG) were used. Data were normalized to glyceraldehyde-3-phosphate dehydrogenase and expressed as the fold change of relative expression.

Statistical analysis

Differences between 2 data sets were analyzed by using the 2-tailed Student *t* test and ANOVA with GraphPad Prism software (GraphPad Software, La Jolla, Calif).

RESULTS

Selective antigen targeting to immature LCs and CD103⁺ DCs

Targeting antigen to LCs by using anti-mouse langerin mAb delivers antigen to LCs, as well as to CD103⁺ DCs.^{12,20,21} To target antigen to LCs only, we used recombinant fusion protein of a humanized mAb specific for the ectodomain of human langerin (clone 2G3) and peptide epitopes derived from I-E₅₂₋₆₈ and 2W1S (2G3-Ag).^{3,19} The humanized 2G3 mAb does not cross-react with mouse langerin and showed no unspecific binding to mouse Fc receptors.^{3,19} huLangerin-diphtheria toxin receptor BAC transgenic mice were previously generated to ablate LCs by means of administration of diphtheria toxin.¹⁵ In these mice transgenic human langerin is efficiently expressed by LCs but not by other DCs expressing mouse langerin.¹⁵ Because we are using these mice as huLangerin transgenic mice and not ablating LCs by using diphtheria toxin, they will henceforth be referred to as huLangerin mice. huLangerin and WT littermate control mice

were injected intraperitoneally with 10 μ g of AF647 conjugated 2G3 to confirm the specificity of targeting LCs. LCs isolated from the epidermis had efficiently acquired 2G3-AF647 (see Fig E1, A, in this article's Online Repository at www.jacionline.org). Keratinocytes, dendritic epidermal T cells, and LCs from littermate control mice did not acquire the antibody (see Fig E1, A). Similarly, 2G3-AF647 could only be detected in LCs from LNs harvested from huLangerin and not control mice (see Fig E1, B). No staining was detected in the spleen (see Fig E1, C). Importantly, targeting LCs with 2G3 did not result in LC activation, as determined by measuring surface expression of CD40, CD86, or MHCII (see Fig E2, A and B, in this article's Online Repository at www.jacionline.org). Expression of cytokines and chemokines indicative of DC activation was also unaltered by 2G3 targeting (see Fig E2, C). Thus anti-huLangerin (2G3)-peptide fusion proteins efficiently and selectively target LCs without activating them and therefore mimic langerin-mediated acquisition of antigen during steady-state conditions.

To selectively target antigen to CD103⁺ DCs, we injected huLangerin-diphtheria toxin subunit A mice ($LC^{-/-}$) that lack LCs²² with anti-muLangerin antibody conjugated to AF647 (4C7).¹⁹ huLangerin-diphtheria toxin subunit A mice, unlike muLangerin-diphtheria toxin receptor mice that have both depleted LCs and CD103⁺ DCs, only have selective absence of epidermal LCs.^{7,22} As shown in Fig E1, D, in the absence of LCs, only CD103⁺ DCs were targeted, and no labeling was observed in the spleen (see Fig E1, E). Thus 4C7 antibody can be used in $LC^{-/-}$ mice to deliver antigen to CD103⁺ DCs.

Steady-state LCs and CD103⁺ DCs expand naive CD4⁺ T cells

To determine the specificity of antigen targeting to LCs, we adoptively transferred CD90.1 CFSE-labeled TE α cells into huLangerin and littermate control (WT) mice. Mice were given 1 μ g of 2G3-E α intraperitoneally. On day 4, TE α cells had expanded approximately 6-fold (Fig 1, A) in huLangerin mice and had completely diluted their CFSE (see Fig E3, A, in this article's Online Repository at www.jacionline.org). TE α cells did not expand in control mice that lacked huLangerin expression. huLangerin-Cre I-A β^{fl} mice have an LC-specific ablation of MHCII but do not express huLangerin because the insertion of *Cre* into the langerin gene in the human BAC disrupted the open reading frame.^{16,23} To test whether LCs directly present antigen to TE α cells, we generated huLangerin $\times$ huLangerin-Cre I-A β^{fl} (from now on $LC^{\Delta\text{MHCII}}$) mice. 2G3-Ag was efficiently targeted to LCs in these mice (data not shown), but TE α cells did not expand (Fig 1, A), indicating that steady-state LCs induce CD4⁺ T-cell proliferation through direct cognate interaction.

It has recently been demonstrated that TCR transgenic CD4⁺ T cells represent the responses of individual TCR clones and are not necessarily indicative of the endogenous T-cell response.²⁴ Thus rather than using adoptive transfer of TCR transgenic T cells to analyze T_H differentiation, we examined the response of endogenous CD4⁺ T cells using an I-A^b:2W1S tetramer/pulldown technique.¹⁴ We immunized WT and huLangerin mice with 1 μ g of 2G3-2W1S intraperitoneally and examined the expansion of 2W1S-specific CD4⁺ T cells using an I-A^b:2W1S tetramer. 2W1S-specific CD4⁺ T cells expanded approximately 10-fold

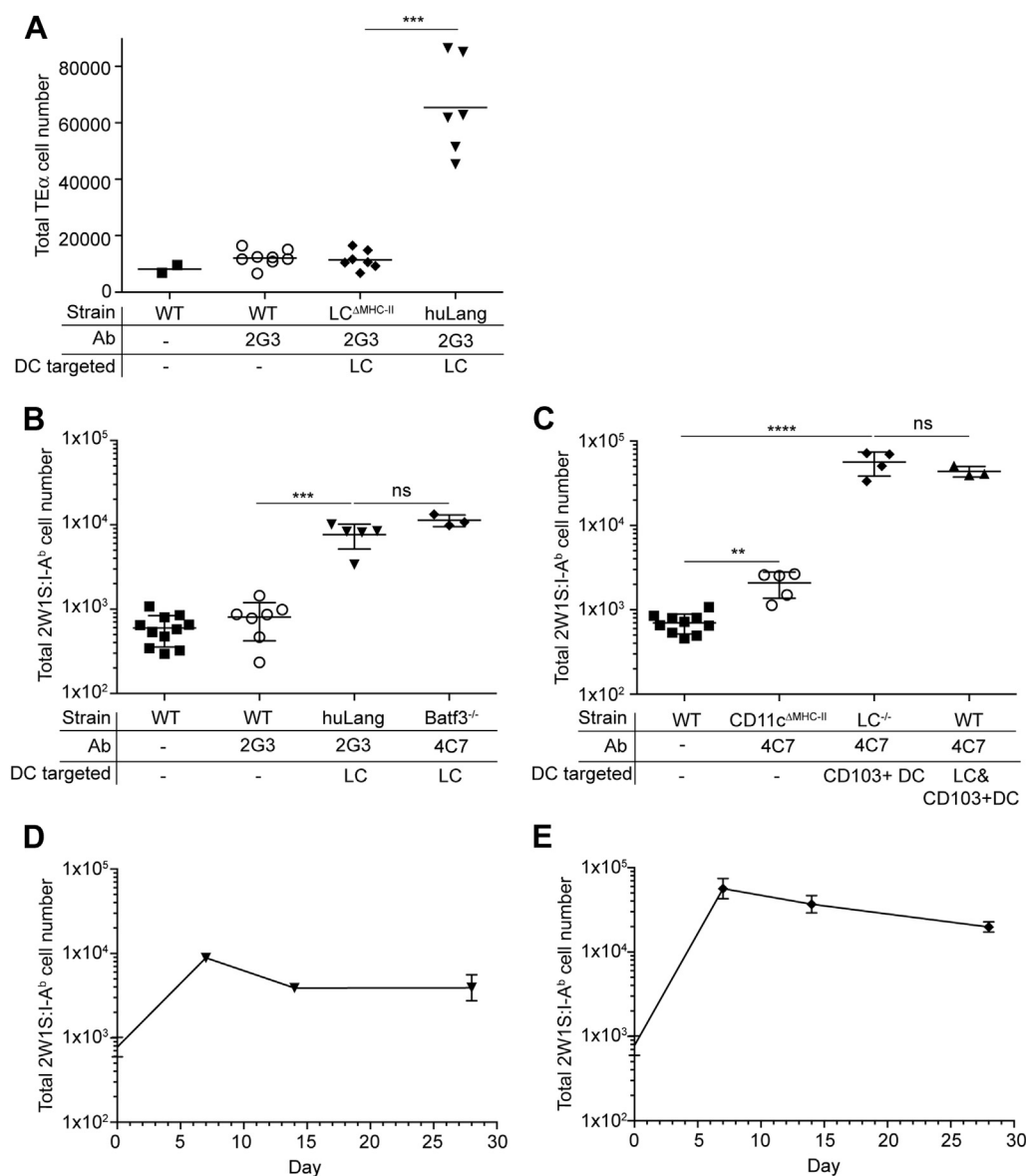


FIG 1. Steady-state LCs and CD103⁺ DCs promote long-lasting CD4⁺ T-cell responses. **A**, TEα cell numbers after 2G3-Eα immunization. **B** and **C**, 2W1S-specific CD4⁺ T-cell responses after 2G3-2W1S or 4C7-2W1S immunization. **D** and **E**, The kinetics of 2W1S-specific CD4⁺ T-cell responses induced by LCs (Fig 1, **D**) or CD103⁺ DCs (Fig 1, **E**). Pooled data of 2 independent experiments are shown. ***P* < .005, ****P* < .0005, and *****P* < .0001. *ns*, Not significant.

in huLangerin mice but did not proliferate in WT mice (Fig 1, **B**, and see Fig E3, **B**).

huLangerin is expressed as a transgene in huLangerin mice.¹⁵ To confirm that targeting endogenous muLangerin in LCs would promote similar CD4⁺ T-cell responses as huLangerin, we immunized Batf3^{-/-} mice with 4C7-2W1S. Because these mice lack CD103⁺ DCs,¹⁷ only LCs would be targeted by anti-muLangerin (4C7) antibody. We found no significant differences in T-cell expansions by targeting endogenous muLangerin or huLangerin (Fig 1, **B**).

To define the T-cell responses induced by steady-state CD103⁺ DCs, we injected WT, CD11c-Cre-MHCII^{fl/fl} (CD11c^{ΔMHCII}), and LC^{-/-} mice with 1 μg of 4C7-2W1S intraperitoneally and examined the expansion of 2W1S-specific CD4⁺ T cells by using

an I-A^b:2W1S tetramer at day 7 after injection. We observed a robust expansion of 2W1S-specific CD4⁺ T cells by CD103⁺ DCs (Fig 1, **C**), which did not differ significantly from responses induced by the joint effort of LCs and CD103⁺ DCs (Fig 1, **C**). In CD11c^{ΔMHCII} mice, in which DCs were not able to present antigen to CD4⁺ T cells, we observed a minimal expansion of 2W1S-specific T cells (Fig 1, **C**). Thus direct presentation of targeted antigen by DCs is required for optimal T-cell responses, and CD103⁺ DCs play a dominant role in induction of CD4 proliferation when both LCs and CD103⁺ DCs are targeted.

To determine the kinetics of CD4⁺ T-cell responses initiated by LCs and CD103⁺ DCs, we immunized huLangerin mice with 1 μg of 2G3-2W1S administered intraperitoneally and LC^{-/-} mice with 1 μg of 4C7-2W1S administered intraperitoneally.

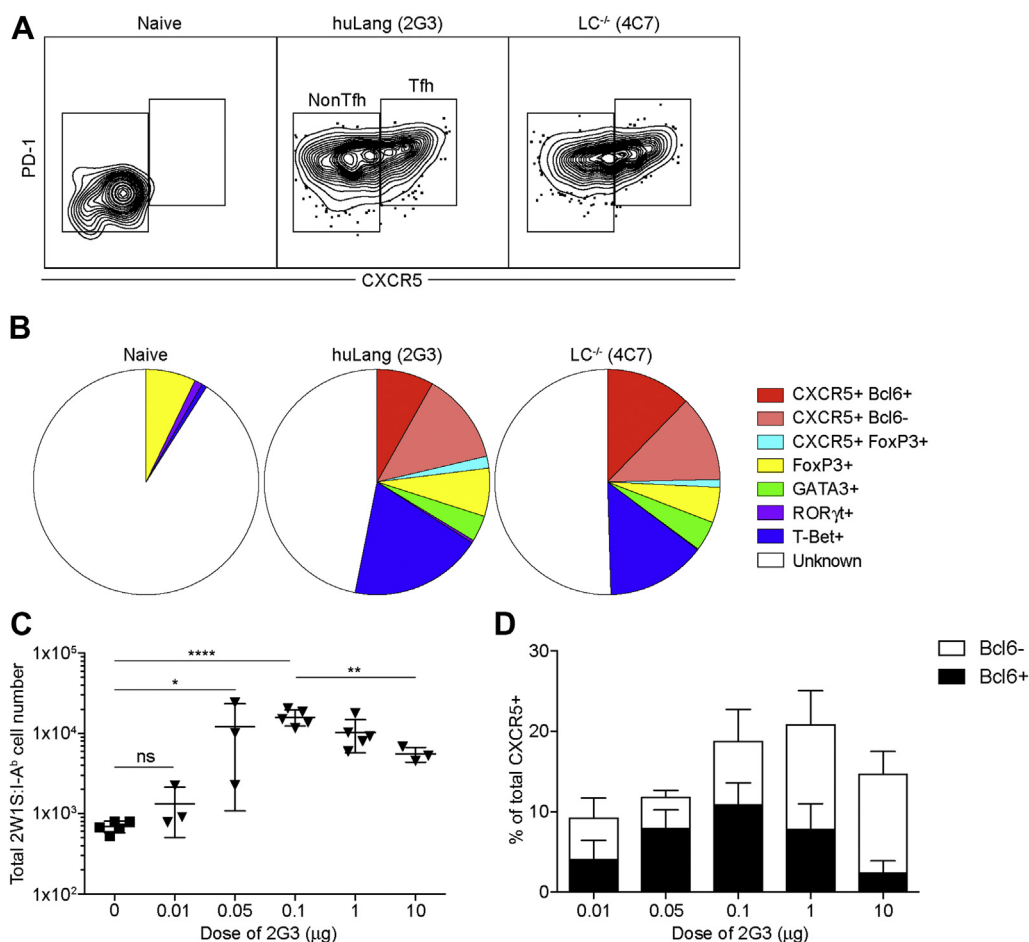


FIG 2. Steady-state LCs and CD103⁺ DCs induce formation of T_{FH} cells in endogenous antigen-specific CD4⁺ T cells. **A**, 2W1S-specific cells stained for PD-1 and CXCR5. **B**, T_H phenotype of 2W1S-specific cells. **C**, Dose response in huLangerin mice. **D**, Cells from Fig 2, C, were stained for the indicated markers. Results are pooled data from 3 independent experiments. **P* < .05, ***P* < .005, and ****P* < .0005. ns, Not significant.

The mice were killed at the indicated time points, and the I-A^b:2W1S tetramer-positive cells were enumerated by means of flow cytometry. T-cell responses induced by both LCs and CD103⁺ DCs peaked at day 7 (Fig 1, D and E). The initial peak was followed by a contraction with no signs of deletion. Thus steady-state skin DCs promote long-lasting CD4⁺ T-cell responses.

Steady-state LCs and CD103⁺ DCs promote differentiation of T_{FH} cells

We next examined the phenotype of 2W1S cells expanded by LCs or CD103⁺ DCs based on expression of PD-1 and CXCR5. We found that 15% to 30% of the responding CD4⁺ T cells expanded by both DCs coexpressed PD-1 and CXCR5, identifying them as T_{FH} cells (Fig 2, A). To further characterize the T_H phenotype, we performed intracellular staining for Bcl-6 (T_{FH} cells), T-bet (T_H1 cells), FoxP3 (Treg cells), Gata-3 (T_H2 cells), and RORγt (T_H17 cells, see Fig E4 in this article's Online Repository at www.jacionline.org). We found that around half of the CXCR5⁺ cells also expressed Bcl-6, which is considered a specific marker of germinal center (GC) T_{FH} cells.^{25,26} We noted no change in the percentage of Treg cells between naive and

immunized mice (*P* > .05). Around 10% to 15% of the cells expressed T-bet, and 3% to 5% were GATA-3 positive. No sizable population of RORγt-expressing cells was detected (Fig 2, B). No significant differences were detected between T_H phenotypes induced by LCs and CD103⁺ DCs (*P* > .05 for all subsets). Thus antigen presentation by LCs and CD103⁺ DCs in the absence of inflammation leads to generation of a mixed T_H cell phenotype dominated by T_{FH} cells.

Recently, it has been shown that antigen dose can influence T_{FH} cell differentiation.^{24,27,28} To determine whether induction of cells with the T_{FH} phenotype by LCs depends on antigen dose, we immunized huLangerin mice with increasing amounts of 2G3-2W1S, ranging from 10 ng up to 10 μg. The cell expansion (Fig 2, C) was determined as described above. At the lowest dose (10 ng), we observed a minimal expansion (≤2 fold) of the 2W1S-specific CD4⁺ T cells. The optimal dose was between 100 ng and 1 μg, with 12- to 15-fold expansion. At the highest dose measured (10 μg), the expansion started to decrease significantly. To test whether the antigen dose affected T_{FH} cell differentiation, we stained the cells for CXCR5/PD-1 and Bcl-6. We observed T_{FH} cell induction by LCs with different efficiencies at all doses tested (Fig 2, D). These data support the idea that T_{FH} induction by LCs is not due to a high antigen dose.

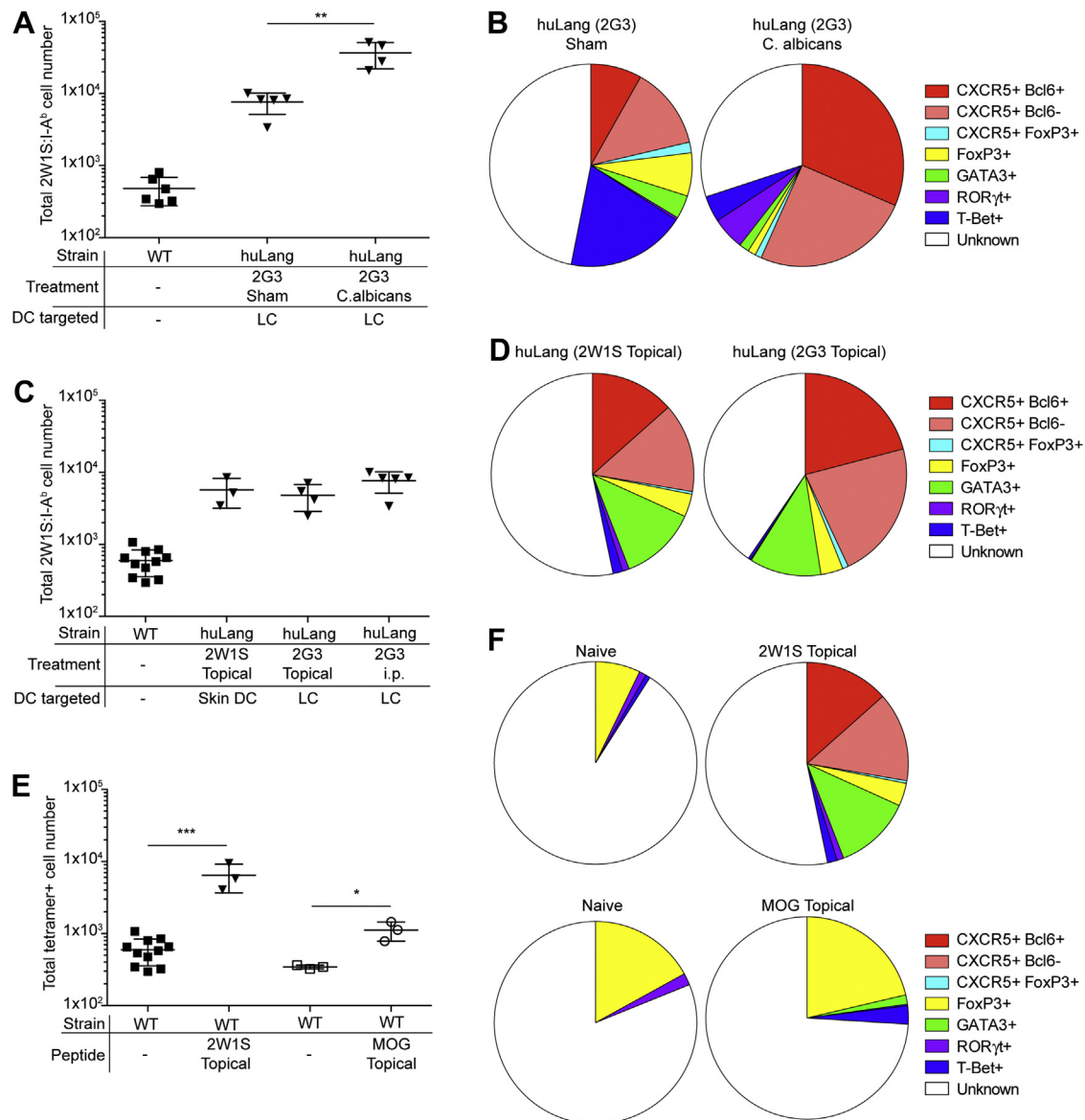


FIG 3. T_H cell induction by skin DCs is not unique to steady-state targeting but is limited to foreign antigen. **A-D**, Total numbers of 2W1S-specific T cells or their T_H phenotype in different conditions. **E** and **F**, Total 2W1S- and MOG-specific T-cell numbers (Fig 3, **E**) and their T_H phenotype (Fig 3, **F**). Results are pooled data from 3 independent experiments. **P* < .05, ***P* < .005, and ****P* < .0005. ns, Not significant.

LCs also promote T_{FH} cell generation in inflammatory conditions

Previously, we reported that targeting 2G3-Eα to LCs during *C. albicans* infection resulted in expansion of T_H17 cells.³ To test whether LCs induce T_{FH} cells in addition to T_H17 cells, we immunized huLangerin mice with 1 μg of 2G3-2W1S administered intraperitoneally and infected the skin with WT *C. albicans*. Seven days later, CD4⁺ T-cell responses were assessed, as described above. *C. albicans* infection combined with targeting induced a more robust T-cell response than targeting alone (Fig 3, **A**). *C. albicans* infection also had a significant effect on the T_H phenotype. Compared with targeting alone, *C. albicans* induced RORγt expression in naive CD4⁺ T cells (*P* < .05) and a more robust T_{FH} cell response (>50%, *P* < .005; Fig 3, **B**). CD103⁺ DCs also induced T_{FH} cells during *C. albicans* infection (see Fig E5

in this article's Online Repository at www.jacionline.org). Thus LCs and CD103⁺ DCs can promote T_{FH} cell responses during inflammatory conditions, and the T_H phenotype is dependent on adjuvant.

T_{FH} cell induction by LCs is not unique to targeting

To determine whether T_{FH} cell induction by LCs occurs only with langerin-targeted antigen, we applied 2W1S peptide or 2G3-2W1S topically on the skin of wet-shaved WT and huLangerin mice, respectively. Topical application of 2G3-2W1S specifically targets epidermal LCs and not LN LCs (see Fig E6 in this article's Online Repository at www.jacionline.org). We found that topical application of 2W1S and 2G3-2W1S promoted T-cell responses comparable with those seen with intraperitoneal targeting (Fig 3, **C**). We also observed robust T_{FH} cell induction with

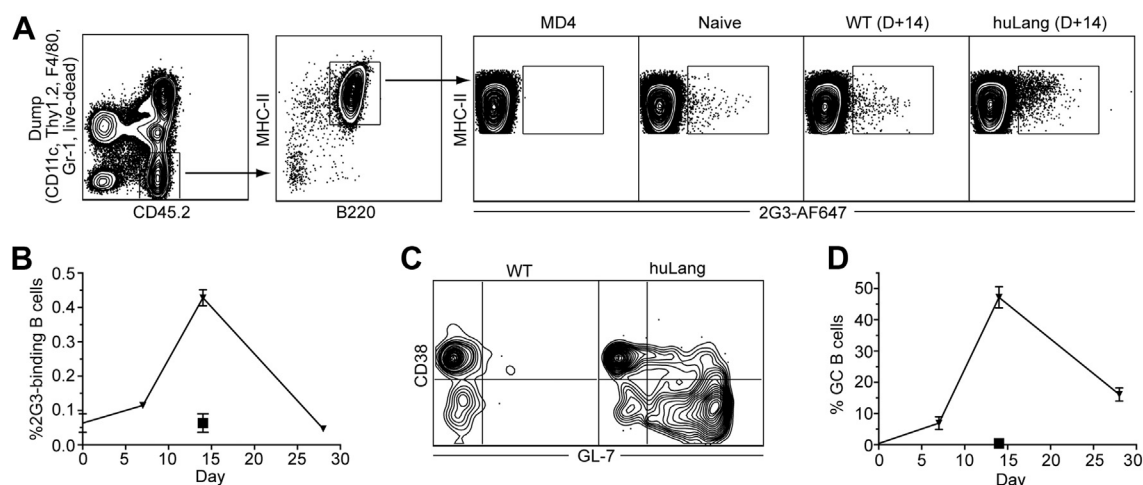


FIG 4. LCs promote B-cell responses. **A**, Gating strategy to identify 2G3-binding B cells. **B**, Kinetics of 2G3-specific B-cell responses in WT (square) and huLangerin (triangle) mice. **C**, Phenotype of 2G3-specific B cells 14 days after immunization. **D**, As in Fig 4, B, except that the kinetics of GC B cells (CD38⁺ GL-7⁺) are shown. Pooled data from 3 experiments are shown.

topical application of the antigen and, unlike with intraperitoneal targeting, minimal T-bet expression and a sizable Gata-3 population ($P < .05$; Fig 3, D). Thus these data suggest that T_{FH} cell induction by skin DCs is not unique to targeting, and that epidermal LCs and LN LCs might induce different responses, Gata-3 and T-bet, respectively.

Skin DCs do not promote T_{FH} cell responses against MOG

Targeting self MOG peptide to langerin-positive DCs resulted in induction of FoxP3 in the adoptively transferred MOG-specific transgenic CD4⁺ T cells and provided resistance to EAE development.¹² Thus it was unexpected that targeting foreign antigen to skin DCs by using a similar technique yielded T_{FH} cells and no significant expansion of Treg cells in our system. To reconcile this obvious discrepancy, we topically applied either 2W1S (foreign) or MOG (self) peptide to wet-shaved WT mice. Seven days later, we assessed the endogenous T-cell responses using 2W1S- and MOG-specific tetramers. We found that 2W1S-specific CD4⁺ T cells expanded approximately 10-fold over naive numbers, whereas the MOG-specific T cells expanded 2- to 3-fold (Fig 3, E). Painting of 2W1S showed the already documented phenotype, with robust T_{FH} cell induction. However, presentation of MOG by skin DCs did not induce T_{FH} cells but rather selectively expanded the FoxP3⁺ Treg cell population (Fig 3, F). Thus this likely reflects differences in the endogenous T-cell repertoire to a foreign antigen (2W1S) versus self-antigen (MOG).

Bcl-6⁺ T_{FH} cells are B-cell dependent but independent of IL-6 and type I interferon

The differentiation of T_{FH} cells is B-cell dependent and relies on the presence of inflammatory cytokines, such as IL-6 and type I interferon.²⁹ The requirements of T_{FH} cell induction in the absence of adjuvants have never been addressed. To test the role of B cells, IL-6, and type I interferon in the generation of T_{FH} cells in homeostatic conditions, we immunized μ MT (lack

mature B cells), IL-6^{-/-}, and IFNAR^{-/-} mice with 1 μ g of 4C7-2W1S administered intraperitoneally and analyzed the T-cell responses as presented above. We found that the overall expansion of 2W1S-specific CD4⁺ T cells was not affected by the absence of IL-6 and IFNAR, but B-cell deficiency led to an exaggerated T-cell expansion (see Fig E7, A, in this article's Online Repository at www.jacionline.org). The overall percentage of T_{FH} cells defined by PD-1 and CXCR5 expression was unchanged along the different mouse lines (see Fig E7, B). Interestingly, the percentage of Bcl-6⁺ CXCR5⁺ T_{FH} cells was significantly decreased in mice lacking B cells but not in IL-6^{-/-} and IFNAR^{-/-} mice (see Fig E7, C). Thus DCs are sufficient to expand CXCR5⁺ T_{FH} cells through an IL-6- and IFNAR-independent mechanism, but B cells are required for sustained Bcl-6⁺ expression.

Antigen presentation by LCs promotes B-cell responses

Generation of T_{FH} cells by skin DCs prompted us to characterize the B-cell response. To define the endogenous B-cell responses initiated by LCs, we took advantage of the fact that our targeting antibodies are fully humanized. As such, in mice B cells specific to human epitopes should be present. To test this, we incubated cell suspensions generated from LNs of MD4 (with B-cell receptor specific to HEL), naive, 2G3-immunized WT (D+14), and huLangerin (D+14) mice with 2G3-AF647 *ex vivo*. Antigen-specific B cells were identified as dump negative (Thy1.2, Gr-1, CD11c, F4/80, and Live/Dead), CD45⁺, MHCII⁺, B220⁺, and AF647⁺ events (Fig 4, A). No binding of 2G3-AF647 to MD4 cells was observed. We found that in a naive mouse around 0.05% of total B cells bound specifically to 2G3-AF647 and that the 2G3-specific B cells did not expand in littermate WT control mice (Fig 4, A and B). However, as expected, in immunized huLangerin mice we observed a 10-fold expansion of 2G3-specific B cells (Fig 4, A and B). To determine the kinetics of the B-cell responses, we immunized WT and huLangerin mice with 1 μ g of 2G3 administered intraperitoneally. The mice were killed at the indicated time points, and the percentage of

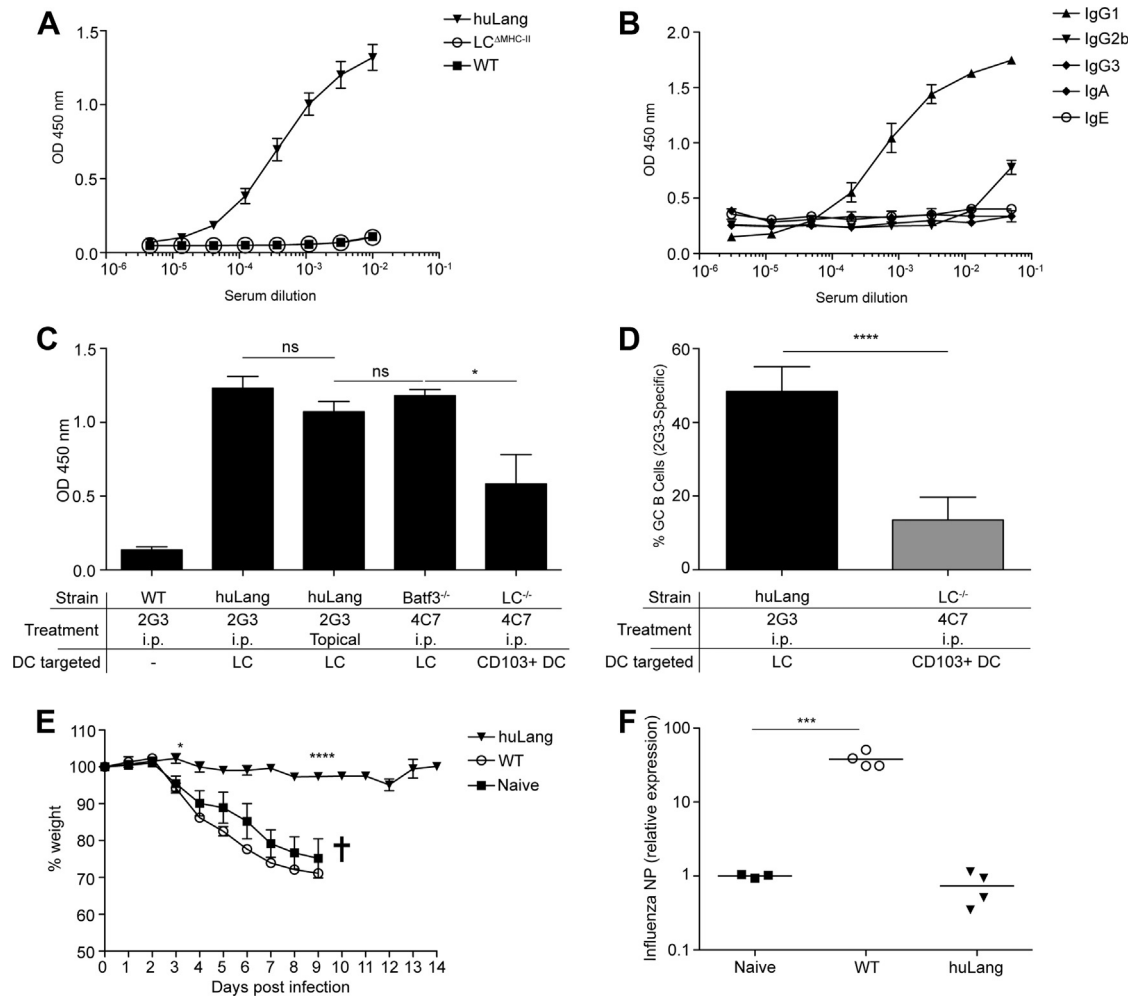


FIG 5. Skin DCs induce humoral immune responses. **A**, Total 2G3-specific IgG levels in serum samples from WT, LC^{ΔMHCII}, and huLangerin mice were determined by means of ELISA. **B**, Serum samples from Fig 5, **A**, were analyzed for isotypes. **C**, Total IgG levels at 1:100 dilution. **D**, Percentage of 2G3-specific GC B cells. **E** and **F**, Weight loss (Fig 5, **E**) and viral load (Fig 5, **F**). Pooled data from 2 independent experiments are shown. * $P < .05$, *** $P < .0005$, and **** $P < .0001$. ns, Not significant.

2G3-specific B cells was determined by means of flow cytometry. B-cell responses peaked around day 14, followed by a contraction (Fig 4, **B**). To test whether antigen presentation by LCs leads to GC B-cell formation, the 2G3-specific B cells were stained for memory/naïve (CD38) and GC markers (GL-7). Antigen presentation by LCs led to a robust GC B-cell (CD38⁺GL-7⁺) response by day 14 (Fig 4, **C**), which followed similar kinetics as the total B-cell response (Fig 4, **D**). Thus these data suggest that antigen presentation by LCs is necessary for initiation of B-cell responses.

LCs and CD103⁺ DCs promote humoral immune responses

Because T_{FH} cells are critical for humoral immunity, including generation of long-lived, high-affinity plasma cells and memory cells,²⁵ we next examined the humoral immune responses induced by steady-state LCs. To confirm the development of a humoral immune response on antigen targeting to LCs, huLangerin, LC^{ΔMHCII}, and WT mice were immunized with 1 μg of 2G3 administered intraperitoneally and boosted with the same dose

14 days later. A strong anti-2G3 titer was observed in the sera of huLangerin mice, but not LC^{ΔMHCII} and WT mice, 21 days after immunization (Fig 5, **A**). LCs promoted an IgG response that was dominated by IgG₁. Other isotypes, such as IgG₃, IgE, and IgA, except IgG_{2b}, were near the level of detection (Fig 5, **B**). Thus steady-state LCs promote IgG₁ humoral immune responses through cognate interaction with CD4⁺ T cells.

To compare humoral immune responses induced by epidermal LCs versus LN LCs, we immunized huLangerin mice through either topical application of 2G3 or intraperitoneal injection. To assess the humoral immune responses triggered by targeting huLangerin versus muLangerin, we injected Batf3^{-/-} mice with 4C7. We found similar antibody responses in all 3 cases (Fig 5, **C**). Thus epidermal and LN LCs promote comparable humoral immune responses independent of the nature of the langerin targeted.

To define the antibody responses initiated by CD103⁺ DCs, we immunized LC^{-/-} mice with 4C7. We found that CD103⁺ DCs also promoted humoral immune responses but were less efficient than LCs (Fig 5, **C**). Consistent with lower antibody levels,

CD103⁺ DCs induced significantly fewer GC B cells (Fig 5, D). Antibody isotypes induced by CD103⁺ DCs did not differ significantly from those induced by LCs (data not shown). Thus steady-state LCs and CD103⁺ DCs promote similar humoral immune responses, but LCs are more efficient than CD103⁺ DCs.

LCs promote protective humoral immune responses

The hallmark of humoral immune responses that involve T_{FH} cells is the presence of long-lived protective antibodies.²⁵ Therefore we sought to determine whether steady-state LC-promoted humoral immune responses could confer systemic protection against a viral infection. LCs, unlike CD103⁺ DCs, do not cross-present the targeting construct to CD8 T cells,^{3,5} and therefore the role of antibodies in protection can be directly assessed. Because LCs also promote a dominant IgG₁ response to 2G3-FluHA conjugate (and data not shown),¹⁹ we decided to assess the efficiency of the humoral immune response induced by LCs in an influenza PR8 (H1N1) infection model. For this purpose, we immunized huLangerin and WT mice with 1 μg of 2G3-FluHA1 administered intraperitoneally. Fourteen days later, the mice were boosted, and 7 days later, the mice were challenged intranasally with 2 × 10⁴ plaque-forming units of live PR8 influenza virus. Naive and WT mice immunized with 2G3-FluHA1 lost approximately 25% of their weight during the course of the first week after infection (Fig 5, E). In contrast, the immunized huLangerin mice did not lose weight (Fig 5, E). Consistent with the failure to lose weight, immunized huLangerin mice had an undetectable viral load (Fig 5, F). Thus antigen presentation by LCs induces production of an effective humoral immune response that can confer systemic protection.

DISCUSSION

Herein we have demonstrated that antigen presentation by LCs and CD103⁺ DCs under steady-state conditions promoted a robust expansion of endogenous antigen-specific CD4⁺ T cells. Antigen targeted by anti-langerin antibody/antigen conjugates and topical application of peptide or protein efficiently induced T_{FH} cell differentiation. T_{FH} cell expansion was further increased by a concomitant *C albicans* skin infection. Contrary to our expectations, significant FoxP3⁺ Treg cell expansion was not detected to a foreign peptide (2W1S), but the self-peptide (MOG₃₅₋₅₅) did expand a population of Treg cells. We also found that skin DCs were sufficient to expand CXCR5⁺ T_{FH} cells through an IL-6- and IFNAR-independent mechanism, but B cells were required for sustained Bcl-6⁺ expression. The expansion of T_{FH} cells by LCs was accompanied by activation and expansion of antigen-specific B cells and development of a robust antibody response that provided systemic protection against influenza infection. Despite inducing more robust CD4⁺ T-cell responses than LCs, the CD103⁺ DCs were less effective in induction of GC B cells and humoral immune responses.

Antigen presentation by LCs has been suggested to result in suppressive T-cell responses and to promote peripheral tolerance.⁸⁻¹⁰ Targeting MOG antigen to langerin-expressing cells resulted in a large expansion of MOG-specific FoxP3⁺ Treg cells and provided resistance to EAE development.¹² Thus it was unexpected that targeting antigen selectively to either LCs or CD103⁺ DCs by using a similar technique did not yield increased Treg cell

numbers in our system. In a side-by-side comparison we found that skin DCs promoted T_{FH} cell responses against 2W1S but not MOG. This likely reflects differences in the endogenous T-cell repertoire to a foreign antigen (2W1S) versus self-antigen (MOG).

Langerin is an antigen uptake receptor that binds a wide variety of ligands and can mediate uptake of numerous pathogens, including HIV.³⁰ We found that targeting langerin with antibody did not induce activation of langerin-expressing cells. We also showed that targeting antigen through transgenically expressed huLangerin and endogenous muLangerin promoted similar T_H responses in LCs. Notably, the topical application of foreign antigen without targeting led to T_{FH} cell induction by skin DCs, arguing that T_{FH} cell induction to skin antigens appears independent of langerin targeting.

The differentiation of T_{FH} cells in infectious models depends on the availability of inflammatory cytokines, such as IL-6 and type I interferons.^{25,26,29} Little is known about the requirements of T_{FH} cell differentiation in the absence of inflammatory signals. How langerin-expressing skin DCs and splenic DCs³¹ promote T_{FH} cells remains to be determined. We did not observe maturation of skin DCs on targeting, as measured based on costimulatory markers and cytokine expression, suggesting that upregulation of these factors might not be required for T_{FH} cell induction in homeostatic conditions. We also noted that the absence of IL-6, IFNAR, or cohousing with conventional mice (data not shown) does not affect induction of T_{FH} cells in our system. Thus it is tempting to hypothesize that T_{FH} cell induction by steady-state DCs reflects a default pathway that is initiated against foreign antigens, which can be further augmented by the presence of inflammatory cytokines. The maintenance of Bcl-6 expression in T_{FH} cells was dependent on the presence of B cells. However, the mechanism of how B cells regulate Bcl-6 expression in T_{FH} cells is not completely understood. A role for inducible costimulator ligand on GC B cells to maintain Bcl-6 expression in T_{FH} cells has been shown.^{32,33} Another interesting candidate to regulate Bcl-6 expression in T_{FH} cells could be B cell-derived IL-10. This cytokine has been shown to be critical to the B cell help functions of T_{FH} cells³⁴; it will be interesting to address whether the reverse could also be true and whether IL-10 is a key tolerogenic cytokine supporting T_{FH} cell development under steady-state conditions.

Presentation of antigen acquired by B cells was initially thought to be the key event driving differentiation of naive CD4⁺ T cells toward the T_{FH} cell lineage.²⁵ More recently, however, T_{FH} cell induction has also been shown to depend on antigen presentation by DCs as well.^{33,35,36} In our model T_{FH} cell expansion and humoral immune responses were absent in control mice lacking expression of huLangerin and in huLangerin-positive mice in which LCs lacked expression of MHCII. In these mice antigen-specific B cells had access to the antigen through direct binding to the B-cell receptor, but they were not sufficient to initiate T_{FH} cell differentiation, which is in accordance with recently published data.³⁷ However, targeting antigen to skin DCs in μMt mice resulted in a significant decrease in Bcl-6⁺ T_{FH} cell numbers, supporting the role of B cells in terminal differentiation of T_{FH} cells. Thus although B cells can sustain Bcl-6⁺ expression, antigen presentation by skin DCs to naive T cells is sufficient for initiation of T_{FH} cell differentiation. Whether direct antigen acquisition through the B-cell receptor, antigen transport/presentation by DCs, or both contributes to B-cell responses remains to be determined. Topical application of antigen triggered

humoral responses comparable with those seen with intraperitoneal targeting. With topical application, antigen availability is limited to skin DCs. As such, active antigen transport, presentation, and possible activation of B cells by DCs should take place. Therefore these data support a model in which DCs, in addition to inducing T_{FH} cells, also regulate humoral immune responses by transporting and presenting antigen to B cells.

LCs and CD103⁺ DCs promote opposing T_H responses against *C albicans*,³ and different antibody isotypes in a gene gun immunization model.³⁸ Here we report that selective antigen targeting to LCs or CD103⁺ DCs in steady state induced comparable T_H phenotypes and antibody isotypes. However, it is also worth noting that CD103⁺ DCs promoted a more robust CD4⁺ T-cell response than LCs but were less effective in inducing antibody production. Consistent with this, CD103⁺ DCs also promoted a very weak GC B-cell response in comparison with LCs. Why these 2 DCs induce different B-cell responses remains to be determined. Multiple things could contribute, such as differences in numbers, localization, and antigen storage. Therefore these data suggest that DCs, in addition to promoting T_H responses, play a direct and important role in B-cell responses, which should be taken into consideration during vaccine design.

The ability of LCs to promote both T_{FH} cell induction and humoral immune responses raises the possibility that these cells might be specialized to generate antibody responses specific for cutaneous antigens, such as commensal microorganisms and/or skin pathogens and their products. We further speculate that LCs might participate in the pathogenesis of antibody-mediated cutaneous diseases, such as pemphigus and allergic dermatitis.

The skin is a common site for immunization, and DC vaccination with anti-lectin/antigen conjugates is emerging as a powerful new vaccination strategy.³⁹ Importantly, our model uses targeting of antigen with anti-human langerin antibody/antigen conjugates in a human langerin transgenic mouse. This is a pre-clinical model for anti-human langerin targeting that is currently undergoing testing in nonhuman primates with the same reagent in anticipation of upcoming clinical trials. Thus in addition to revealing an unexpected DC biology, our findings suggest that targeting antigen to skin DCs either through systemic or topical immunization without adjuvants will be an efficient approach to generate protective antibody responses.

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Clinical implications: Our findings suggest that targeting foreign antigen without adjuvants to a specific skin DC subset either through systemic or topical application will be an efficient approach to generate protective, antibody-based vaccines.

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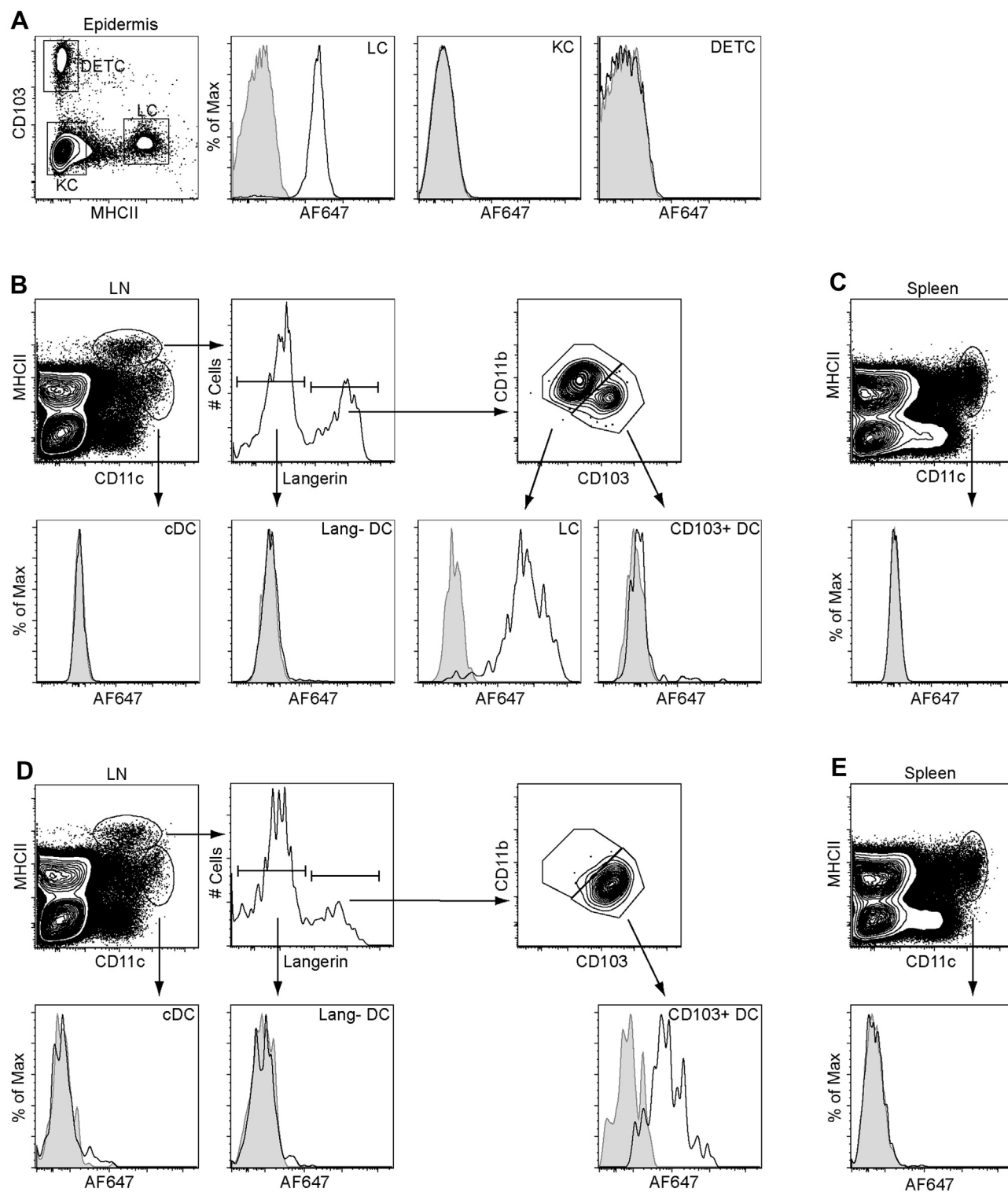


FIG E1. Antigen targeting to LCs and CD103⁺ DCs is specific. The gating strategies are shown on the figures (upstream gate: live/singlets). **A-C**, Shaded areas represent WT and black lines represent huLangerin mice treated with 2G3-AF647. **D and E**, $LC^{-/-}$ mice left untreated (shaded) or injected (black line) with 4C7-AF647. One representative experiment of 3 is shown. DETC, Dendritic epidermal T cells; KC, keratinocytes.

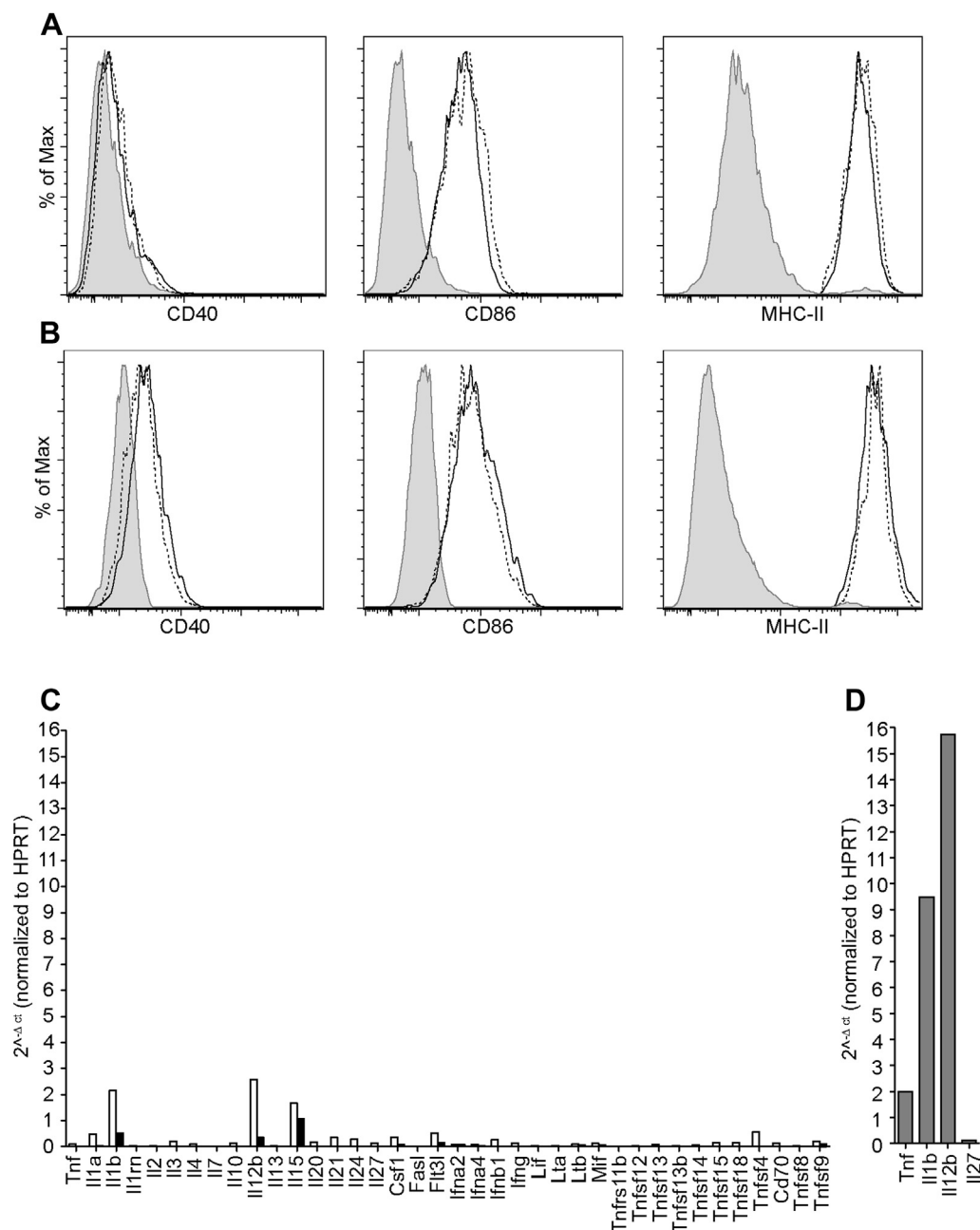


FIG E2. Targeting LCs through langerin does not induce maturation or alter cytokine profile. **A** and **B**, WT (black lines) and huLangerin (dashed lines) mice. Shaded lines indicate isotype controls. **C**, As in Fig E2, A, except that LCs were sorted from LNs of WT (open bars) and huLangerin (black bars) mice and cytokine profile tested. **D**, Cytokine profile of LCs sorted from *C. albicans*-infected mice. HPRT, Hypoxanthine phosphoribosyltransferase.

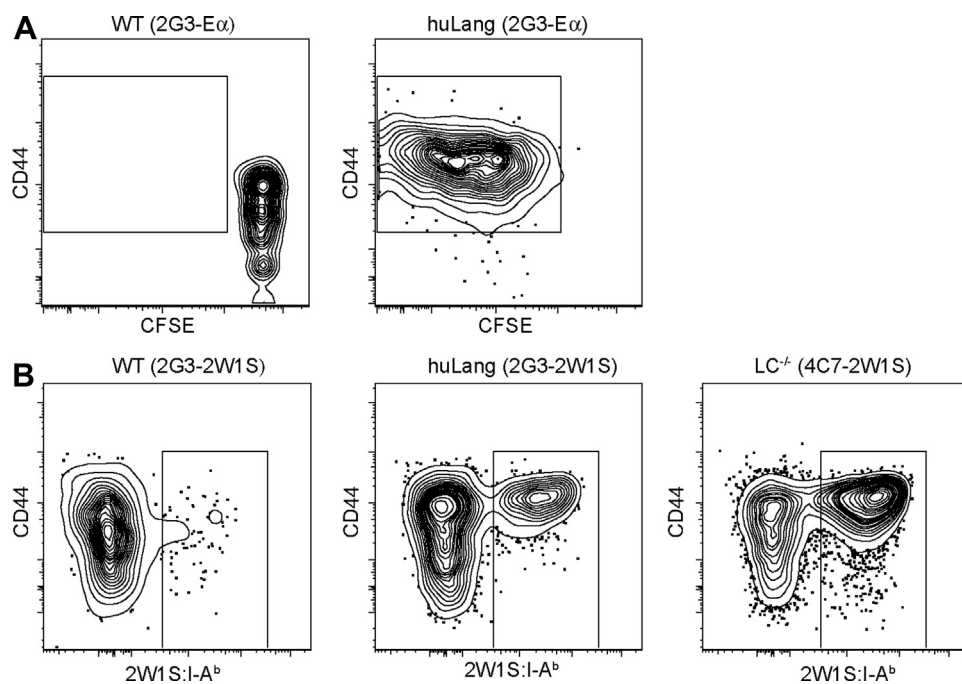


FIG E3. Antigen targeting to LCs and CD103⁺ DCs induce antigen-specific T-cell proliferation. **A**, TE α proliferation profile in WT and huLangerin mice immunized with 2G3-E α . **B**, Endogenous 2W1S-specific T-cell expansion induced by LCs or CD103⁺ DCs.

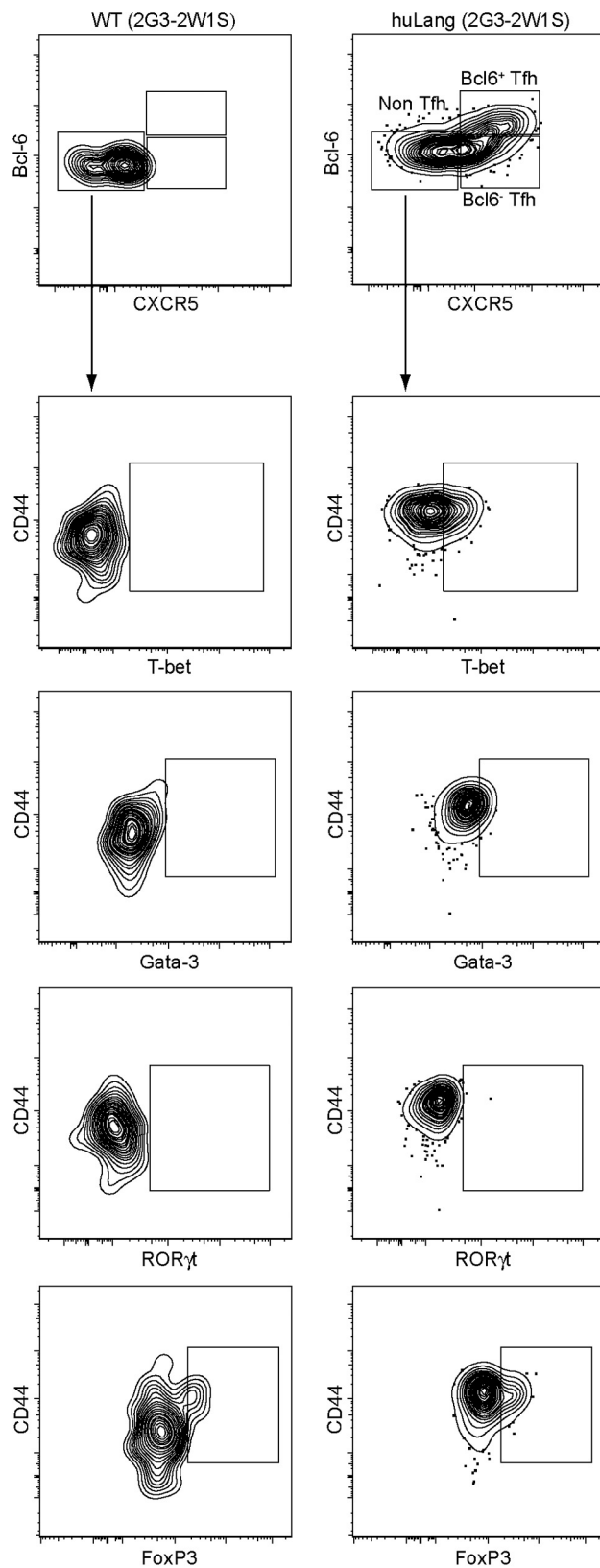


FIG E4. Transcription profile of LC-expanded 2W1S-specific cells.

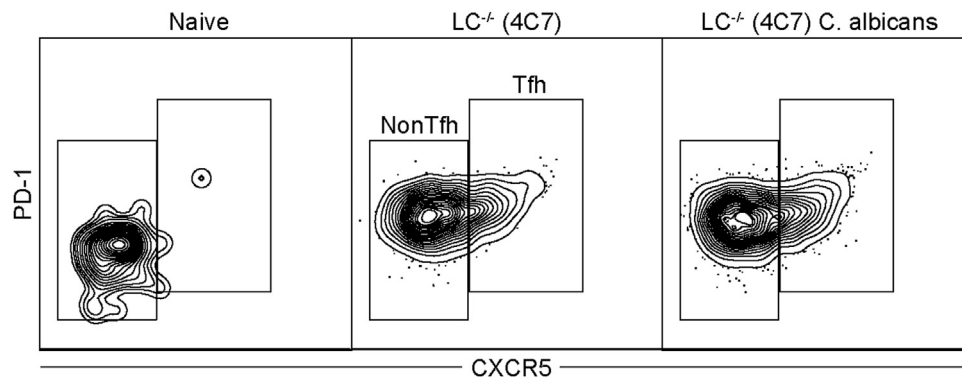


FIG E5. CD103⁺ DCs induce T_{FH} cells in the context of *C. albicans* infection.

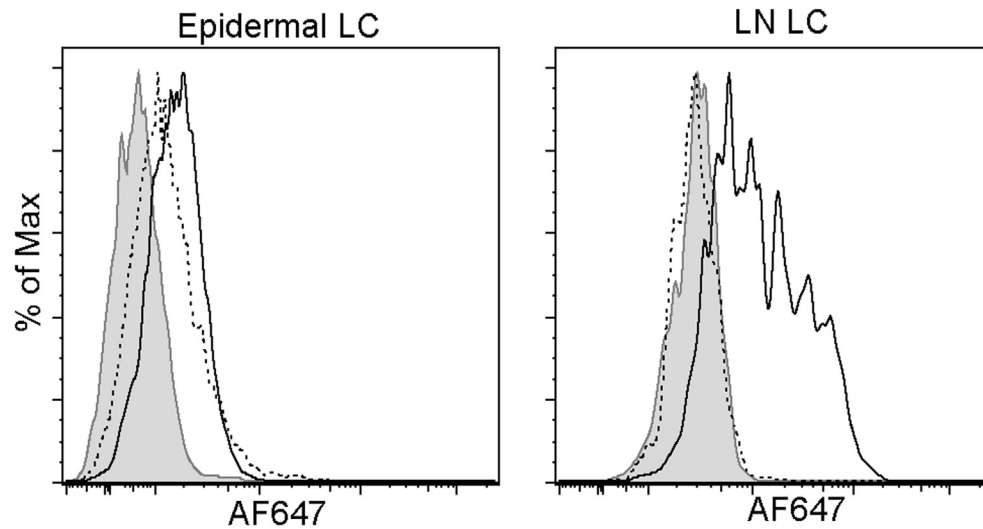


FIG E6. Epidermal application of 2G3-AF647 targets LCs in the epidermis but not LN LCs. Mice were left untreated (*gray shading*), painted with 2G3-AF647 (*dashed line*), or injected intraperitoneally with 2G3-AF647 (*black line*). Skin and LNs were harvested 18 hours after treatment, and the AF647 signal was assessed in LCs.

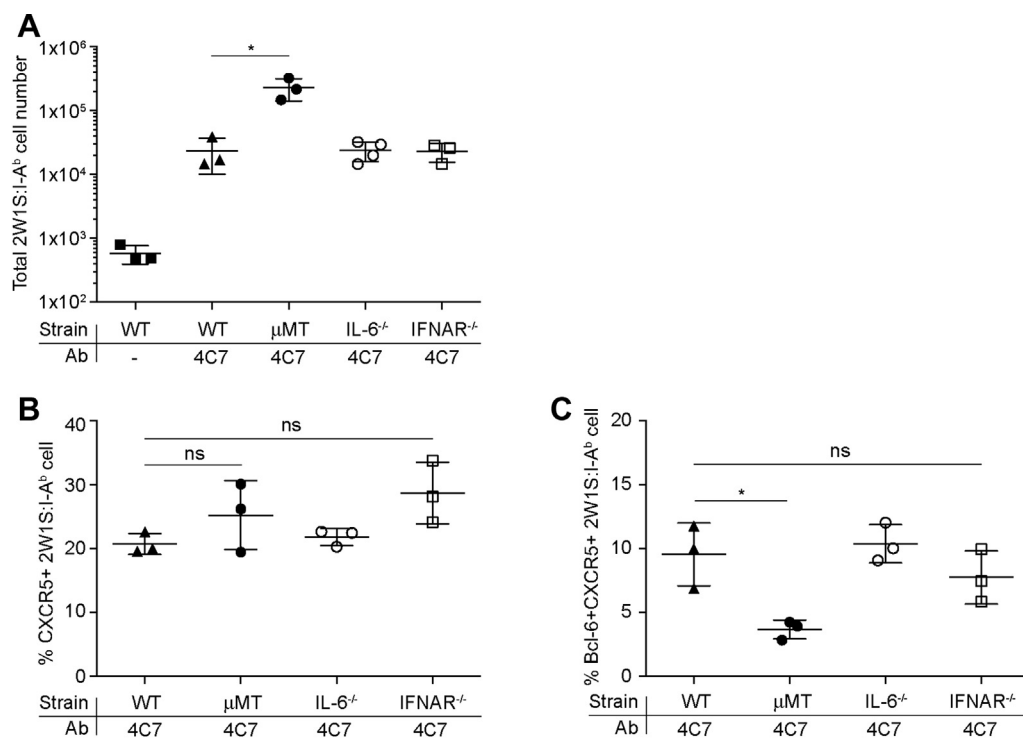


FIG E7. B cells are required for Bcl-6 expression by T_{FH} cells but not IL-6 and type I interferon signaling. μ MT, IL-6^{-/-}, and IFNAR^{-/-} mice were treated with 4C7-2W1S. Seven days later, the expansion of 2W1S-specific cells (**A**) and phenotypes (**B** and **C**) were determined by means of flow cytometry. One representative experiment of 2 is shown. * $P < .05$. ns, Not significant.