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Neoantigen Expression in Steady-State Langerhans Cells Induces CTL Tolerance

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The skin hosts a variety of dendritic cells (DCs), which act as professional APC to control cutaneous immunity. Langerhans cells (LCs) are the only DC subset in the healthy epidermis. However, due to the complexity of the skin DC network, their relative contribution to either immune activation or immune tolerance is still not entirely understood. To specifically study the function of LCs in vivo, without altering the DC subset composition in the skin, we have generated transgenic mouse models for tamoxifen-inducible de novo expression of Ags in LCs but no other langerin* DCs. Therefore, this system allows for LC-restricted Ag presentation to T cells. Presentation of nonsecreted OVA (GFPOVA) by steady-state LCs resulted in transient activation of endogenous CTL in transgenic mice. However, when these mice were challenged with OVA by gene gun immunization in the contraction phase of the primary CTL response they did not respond with a recall of CTL memory but, instead, with robust Ag-specific CTL tolerance. We found regulatory T cells (Tregs) enriched in the skin of tolerized mice, and depletion of Tregs or adoptive transfer of the Tregs resulted in either robust CTL tolerance or CTL memory, and this decision-making depends on the activation state of the presenting DCs. The Journal of Immunology, 2017, 199: 000–000.

Given the prominent anatomic location in the outermost skin layer and mucosal tissues, epidermal Langerhans cells (LCs) have long been regarded as the principal APC subset. Indeed, mostly from in vitro studies, it is well known that LCs are able to present and cross-present Ags to T cells (1–4). In vivo, however, direct functional investigation of LCs in the induction or control of CTL responses, which are essential for viral and tumor defense, is difficult because murine skin accommodates at least four additional dendritic cell (DC) subpopulations in the dermis. Of these dermal DC subsets, two express the C-type lectin langerin/CD207, originally described as a unique marker for LCs, and two lack the expression of langerin (5–10).}

Transgenic mouse models have provided valuable tools for the investigation of Ag presentation by DCs and its consequences on induction of protective immunity or immunological tolerance. For example, transgenic mice expressing the model Ag OVA under the control of keratinocyte-specific promoters have significantly contributed to our knowledge of autoimmunity and tolerance against skin-borne Ags (11–13). Moreover, a mouse model for inducible expression of viral Ags in DCs demonstrated that protective immunity or tolerance against viral Ags depends on the activation state of the presenting DCs (14). However, in these models, the Ags were expressed either in CD11c+ DCs or keratinocytes and thus did not allow for experimental restriction of Ag presentation to epidermal LCs. Models of langerin promoter-driven transgene expression, such as LangEGFP knock-in mice, have substantially contributed to elucidating the dynamics and function of langerin* DCs in vivo (15). In this model, GFP is constitutively expressed throughout life, and therefore does not reflect neoantigens that the organism encounters later in life (e.g., in the course of infections or emerging altered self-antigens).

Finally, mouse models for subset-specific depletion of langerin* DCs have been crucially involved in the identification the langerin* dermal DCs and their superior cross-presenting capacity (16, 17). Moreover, these depletion models severely challenged the view of LCs as the most potent inducers of T cell immunity and uncovered the unexpected regulatory/suppressive role of LCs in vivo. Different mechanisms, including anergy and clonal deletion of T cells (18), IL-10 secretion (19), incomplete maturation (20), or the generation of regulatory T cells (Tregs) (18, 21) have been demonstrated for LC-mediated tolerance induction. However, because LCs were absent in all these systems, the conclusions drawn from the respective studies were inevitably indirect in nature.

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Abbreviations used in this article: cdc, coding sequence; DC, dendritic cell; JGα, β galactosidase; GF, gene gun; hu, human; LC, Langerhans cell; MHC-I, MHC class I; pClO-ova, CMV promoter-driven OVA plasmid; poly(I:C), polyinosinic-polycytidylic acid; RMC, recombinase-mediated cassette exchange; SDLN, skin-draining lymph node; TAM, tumor-associated; Treg, regulatory T cell; WT, wild type.

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The tolerogenic potential of LCs was recently shown in an immunization setting in wild-type (WT) mice. In this study, targeting of OVA selectively to LCs using an OVA-coupled anti-Langerin Ab resulted in robust cross-tolerance rather than in long-lasting cytotoxic immunity against the Ag (22).

In the current study, we aimed at investigating the in vivo function of LCs on CTL immunity against endogenous LC-borne Ags without altering the DC subset composition of the skin. To this end, we have developed transgenic mice in which the expression of Ags, such as OVA, Escherichia coli β galactosidase (βGal) and others is 1) strictly confined to LCs, 2) in a time-controllable manner, 3) under conditions in which all DC subsets are present in their natural physiological state.

Materials and Methods

Mice

Generation of ROSA26 EGFPOVA mice. The coding sequence (cds) of EGFP (23) was ligated to the 5’ end of full-length OVA. The resulting EGFPOVA fusion construct was subcloned, in reverse orientation with respect to ROSA26 transcription, into the recombine-mediated cassette exchange (RMCE) vector (24) using NruI sites. Within the RMCE vector the reversely oriented EGFPOVA construct is flanked by two sets of WT and mutant (L3) oppositely oriented loxP sites: loxPwtFwd–loxPmutL3 fwd–TATAAAGTCTCCTATACGAAGTTAT-3’ (EGFPOVA (inverted)–loxPwtFwd”loxPmutL3”-loxPwtFwd 5’-ATAACTTCGTTACAGCTACATTATACGAAGTTAT-5’; loxPmutL3: 5’-ATAACTTCGTTACAGCTACATTATACGAAGTTAT-5’ (25)). This enables two consecutive Cre-mediated recombination steps, resulting in loxPmutL3 rev–EGFPOVA “loxPwtFwd” and, finally, inversion and subsequent expression of nonsecreted EGFPOVA. For stable genomic insertion of the transgene cassette into the ROSA26 locus, RMCE-compatible ROSA26 embryonic stem cells (24) were cotransfected with the RMCE-EGFPOVA construct and the pFlp recombinase expression vector. Correctly targeted embryonic cell clones were injected into C57BL/6 blastocysts. Chimeric offspring, referred to as ROSA26-EGFPOVA mice, were bred on a C57BL/6 background. For Cre-inducible EGFPOVA expression in LCs, ROSA26-EGFPOVA mice were crossed with the human (hu) Langerin-CreER2 mouse strain (26). In the latter, the expression of CreER2 is driven by the huLangerin promoter, thus enabling expression of cytosolic ECFP (23) in mouse LCs but no other langerin pos DCs. For Cre-activatable, LC-specific expression of YFP, GFP or E. coli βGal, respectively, huLangerin-CreER2 mice were crossed with 26 reporter mouse lines, harboring floxed STOP cassettes separating the cds for YFP, GFP or lacZ from the promoter (27–29). The respective Cre-activatable birnageneic mice are referred to as LCre-YFP, LCre-GFP, or LCre-lacZ. All mice were on a C57BL/6 background, OT-1 (stock no.: 003831; The Jackson Laboratory) and OT-2 (stock no.: 004194) transgenic mice were on CD45.1. All mice were maintained at the local animal facility under specific pathogen-free conditions.

Ethics statement

Animal experiments were conducted in accordance with EU guidelines 86/609/EWG and national legal regulations (Tierersuchgesetz 2012) and all efforts were made to minimize or avoid suffering. Experiments were approved by the Austrian Ministry of Science, permission number GB 66.01220114-II/30/2011. For terminal analysis, mice were euthanized by cervical dislocation.

Tamoxifen treatment and activation of DCs in vivo

LC-restricted neontagen expression under noninflammatory conditions was induced by i.p. injection of 2 mg tamoxifen (TAM; Sigma-Aldrich, Germany) dissolved in 0.1 ml peanut oil:EtOH (9:1) (14). For simultaneous DC activation, TAM treatment was combined with injection of a mixture of an agonistic anti-CD40 Ab [clone: PGK45 (30)] and polyinosinic-polycytidylic acid [poly(I:C)] (Sigma-Aldrich), 50 µg, each delivered i.d. in four spots of 25 µl each into the abdominal skin.

Gene gun immunization

Gene gun (GG) immunization was performed as previously described (31). Briefly, CMV promoter-driven GG vaccine plasmid constructs were based on the eukaryotic expression vector PCI (Promega, Madison, WI), and have been described elsewhere (32, 33). Unless otherwise noted, GG immunization was performed twice, at 2-wk intervals. One dose comprised two nonoverlapping shots onto the shaved abdominal skin. With each shot, 1 µg of plasmid DNA immobilized onto 0.5 mg gold particles (1.6 µm; Bio-Rad, Richmond, CA) was delivered with pressurized helium gas at 400 pounds per square inch using a Helios GG (Bio-Rad).

Depletion or transfer of CD4+CD25+ T cells

For depletion of CD25+ cells, (LCcre-GFPOVA or LCre-lacZ) mice were injected with 250 µg PC61.5 Ab i.p. (Bio X Cell, Lebanon, VT) 1 d before the first GG immunization and again 1 wk later (i.e., the day before the GG boost). Sustained Treg depletion was verified in peripheral blood and the spleen by flow cytometry. For adoptive transfer experiments, CD4+CD25+ T cells from spleen and skin-draining lymph nodes (SDLN) were isolated by two-step magnetic cell separation (CD4+CD25+ Treg Isolation Kit; Miltenyi Biotec) and injected i.v. into naive C57BL/6 recipients (3 x 106 cells per mouse).

Isolation and characterization of Treg cells in the skin and SDLN of LCre-GFPOVA mice

For detection of endogenous, polyclonal Treg cells in the skin, two spots of abdominal skin from GG-immunized or naive mice were isolated. After removal of fatty tissue, skin was cut into small pieces and digested in 2 ml of an enzyme mixture containing collagenase XI (2 mg/ml), hyaluronidase (0.5 mg/ml), and DNase I (0.1 mg/ml) diluted in C10 medium (RPMI 1640, 1% l-glutamine, 10% FBS, 1% penicillin streptomycin, 1 mM Na pyruvate, 1% Heps, and 1% nonessential amino acids) for 45 min at 37°C during constant shaking. Skin cells were filtered through a 100 µm cell strainer and after washing with cold PBS, stained with anti-mouse CD3-APC (clone I45.2C11; BioLegend), fixable live/dead eFluor 506, anti-mouse CD45-FITC (clone A20), anti-mouse CD4-APC780 (clone GK1.5), anti-mouse CD25 -Pe (clone PC61.5), and intracellularly with anti-mouse Foxp3-eFluor 450 (clone MR1-4) (all from eBioscience). Cells were washed and resuspended in cold PBS/10 mM EDTA to prevent cell aggregation.

To analyze the development of OVA-specific Treg cells in SDLN, one million spleen cells isolated from OT-2 mice (34) were adoptively transferred into LCre-GFPOVA mice, either treated with 1 µg TAM on the same day or left untreated. Then 12 or 15 d after cell transfer, SDLN were isolated and stained with a pistil, and lymphocytes were stained with fixable live/dead eFluor 506, anti-mouse CD45.1-FITC (clone A20), anti-mouse CD4-APC-eFluor780 (clone GK1.5), anti-mouse Vβ 5.1, 5.2-PerPCy5.5 (clone MR1-4), anti-mouse CD25 -Pe (clone PC61.5) and intracellularly with anti-mouse Foxp3-eFluor450 (clone FJK-16S). Cells were washed and resuspended in cold PBS. Treg cells in skin and LN samples were analyzed on a FACSsanto II (Becton Dickinson) using Diva software.

Isolation and characterization of DCs in vivo

DC isolation from tissue specimens was performed as described previously (35). Briefly, LN and thymi were digested with collagenase D (120 µg/ml) and DNase I (120 µg/ml) (both from Roche, Vienna, Austria) in RPMI 1640 medium (Sigma-Aldrich). Whole trunk skin was cut into small pieces and digested with Liberase TM (150 µg/ml; Roche) and DNase I in RPMI 1640 medium. Skin specimens were incubated for 45 min and LN and thymus specimens for 30 min at 37°C with agitation. Cells were filtered through 70 µm nylon mesh filters and washed with PBS. Cells were stained with fixable live/dead eFluor 506, anti-mouse CD45.1- FITC (clone A20), anti-mouse CD4-APC-eFluor780 (clone GK1.5), anti-mouse Vβ 5.1, 5.2-PerPCy5.5 (clone MR1-4), anti-mouse CD25 -Pe (clone PC61.5) and intracellularly with anti-mouse Foxp3-eFluor450 (clone FJK-16S). Cells were washed and resuspended in PBS/10 mM EDTA to prevent cell aggregation. Fluorescent stains and YFP expression in LCs was analyzed by flow cytometry on a FACSsanto II (Becton Dickinson).

In vivo proliferation assay

Spleen cells from OT-1 donor mice (CD45.1) and BL6 WT mice were stained with 3 µM CFSE or eFluor 670 (Molecular Probes), and one million cells from each fraction were injected i.v. into transgenic LCcre-GFPOVA mice, either treated with TAM or left untreated. After 4 d, cells isolated from SDLN were stained with α-CD45.2–PerCP, α-CD4–APC, α-CD8–APC and α-Vβ5.1, 5.2 (all from eBioscience). OT-1 proliferation was analyzed by flow cytometry and fold expansion calculated relative to injected WT cells.
OVA-specific CD8+ T cells from SDLN or spleen were analyzed by MHC class I (MHC-I) pentamer (257–264 SIINFEKL; ProImmune) staining. CTL activity was determined in vivo as described (36). Briefly, syngeneic spleen cells were stained with 5 or 0.3 μM CFSE, respectively. The latter fraction was pulsed with 10 μM CTL peptide (OVA 257–264 SIINFEKL) (37) or βGal 96–103, DAPIYTNV (38) and combined with the first (nonpulsed) fraction. Of this mixture, 3 x 10^6 cells were injected i.v. into recipient mice. After 16 h, splenocytes and SDLN cells were analyzed by flow cytometry, and percentage-specific lysis was calculated from the reduction of peptide-pulsed CFSEhi target cells relative to nonpulsed CFSElo reference cells. Ag-specific IFN-γ-secreting CD8+ T cells were detected by ELISPOT, as described elsewhere (33). In brief, whole splenocytes or LN cells were cultured on ELISPOT plates coated with anti-mouse IFN-γ mAb (clone: AN-18; eBioscience) in the presence or absence of CTL peptide (SIINFEKL or DAPIYTNV). After 24 h, cytokine spots were detected by use of biotinylated anti-mouse IFN-γ mAb (clone: R46A2; BioLegend), followed by streptavidin-HRP and 3-amino-9-ethylcarbazol as a chromogenic substrate. Spots were counted manually from flatbed scans of duplicate wells.

Results
Inducible expression and presentation of LC-restricted neoantigens
All transgenic mouse models for inducible expression of LC-borne neoantigens relied on Rosa26 promoter controlled reporter lines. These lines were crossed with the huLangerin-CreER<sup>T2</sup> mice in which, as in other huLangerin transgenic mice generated in the laboratory of Kaplan and colleagues (26, 39, 40), transgene expression was specific for LCs. Thus, in the resulting double-transgenic mice, Ags such as YFP, GFP, E. coli βgal (lacZ), or nonsecreted GFPOVA (Supplemental Fig. 1) were expressed exclusively in LCs, but no other langerin<sup>+</sup> DC subsets, by TAM-mediated nuclear translocation of the Cre recombinase (Fig. 1A).

First, we studied the TAM-controlled induction of transgene expression in LCs. Because we were unable to track GFP expression in LCs of LCre-GFPOVA or LCre-GFP mice, we monitored YFP expression in LCs of LCre-YFP mice, which carry the identical promoter. As previously shown, repeated TAM treatment of LCre-YFP mice resulted in a robust and sustained expression of YFP exclusively in LCs and no other DC subsets (26). To define a sharp starting point of Ag expression and prevent the potential risk of toxic side effects of repetitive TAM administration (41), we injected mice only once with 2 mg TAM and monitored the onset and the kinetics of YFP expression over 16 d. Two days after TAM treatment we found 10% of langerin<sup>pos</sup>EpCam<sup>pos</sup> LCs in the skin positive for YFP, reaching a plateau of ∼36% on day 8 (Fig. 1B).

With a slight delay, YFP expression was also visible in LCs isolated from SDLN (Fig. 1C). YFP-expressing LCs were rare in the skin and in SDLN of untreated LCre-YFP mice (Fig. 1B, 1C) and, importantly, absent in the thymi of these mice (data not shown), demonstrating a TAM-controlled induction of transgene expression in LCs.

As a functional readout of transgene expression, we measured the proliferation of OT-I CD8<sup>+</sup> T cells. We adoptively transferred CFSE-stained OT-I and WT cells (at a 1:1 ratio) into LCre-GFPOVA mice, either treated with TAM or left untreated and, as a control, into TAM-treated Cre<sup>neg</sup> littermates. Then 4 d later, OVA-specific OT-I cells were analyzed for CFSE dilution and...
expansion relative to WT cells. OT-1 cells in TAM-treated Cre<sup>reg</sup> littermates showed no signs of activation and only a weak OT-1 proliferation (4-fold expansion) was visible in untreated LCre-GFPOV A mice (Fig. 1D), suggesting minor leaky OVA expression. By contrast, we found a strong proliferation (26-fold expansion) of OT-1 cells that had completely lost the CFSE dye in SDLN of TAM-treated LCre-GFPOVA mice.

**Presentation of GFPOVA by steady-state LCs primes endogenous CD8<sup>+</sup> T cells in vivo**

Because of the strong activation of OT-1 cells upon OVA presentation by steady-state LCs, we next investigated whether steady-state LCs would also activate endogenous CTL responses against OVA. Therefore, we treated LCre-GFPOVA mice with TAM or left them untreated and monitored endogenous CD8<sup>+</sup> T cell activation over 6 wk. Basal Cre activity (without TAM) was not sufficient to activate endogenous cytotoxic T cells. By contrast, 2 wk after induction of OVA in LCs, ~3.5% of CD8<sup>+</sup> T cells in SDLN and in the spleen were specific for OVA as revealed by K<sup>B</sup>/SIINFEKL pentamer staining (Fig. 2A, 2B, Supplemental Fig. 2). Moreover, these cells were able to kill ~50% of CTL peptide-loaded target cells in vivo (Fig. 2C, 2D), and secreted IFN-γ upon in vitro restimulation with CTL peptide SIINFEKL (Fig. 2E, 2F). In SDLN, CTL peaked after 2 wk, and after 4 wk in the spleen. In both tissues, 6 wk post-TAM treatment CTLs had strongly declined. Thus, de novo expression of Ags in LCs was sufficient for the initial activation of cytotoxic T cells without the requirement for an additional immune-activating stimulus.

**Neoantigen presentation by steady-state LCs induces CTL tolerance**

CTLs can persist for a long time and acquire a memory function even under suboptimal stimulation conditions (42). Similarly, in our LCre-GFPOVA mice, Ag presentation by resting LCs without additional costimulation was sufficient to prime endogenous CTL, and to develop effector function. We therefore asked whether these cells also might have acquired a recallable memory phenotype. For that purpose, we subjected TAM-treated LCre-GFP/OVA mice to cutaneous GG immunization with a CMV promoter-driven OVA plasmid (pCI-Ova), previously proven to induce robust effector/memory CTL (33, 43). We chose two different time points for the first GG challenge, either at the peak of the primary CTL response (2 wk post-TAM) or during CTL contraction (4 wk post-TAM) (Fig. 2). Mice were GG boosted 2 wk later and, after another 2 wk, analyzed for CTL functions (Fig. 3A). As expected, we found a strong activation of CD8<sup>+</sup> T cells against Ova in both TAM-treated WT and untreated LCre-GFPOVA mice. This was reflected by their capability to eradicate 30–40% of SIINFEKL-pulsed target cells in vivo (Fig. 3B) and SIINFEKL-dependent IFN-γ secretion after restimulation in vitro (Fig. 3C). These effects were strongly amplified in TAM-treated LCre-GFPOVA mice when the GG challenge with pCI-Ova was set at the peak of the primary CTL response (i.e., 2 wk post-TAM). By contrast, when TAM-treated LCre-GFPOVA mice were GG challenged in the contraction phase of the primary CTL response (i.e., 4 wk post-TAM treatment), they were resistant to GG immunization. From this we concluded that despite the initial activation of cytotoxic T cells by resting LCs, a robust CTL tolerance against the neoantigen OVA manifests during the contraction phase of the primary response.

**LC-mediated CTL tolerance is Ag specific**

To confirm that the observed TAM-induced CTL tolerance was Ag specific, LCre-GFPOVA and LCre-GFP transgenic mice were treated with TAM to trigger GFPOVA or GFP expression respectively in LCs. Cre<sup>reg</sup> littermates treated in the same way served as WT controls. After 4 wk, mice were GG challenged with OVA DNA, twice at a 2-wk interval, and were analyzed for OVA-specific CTL functions 2 wk later (Fig. 4A). CD8<sup>+</sup> T cells from TAM-treated LCre-GFPOVA mice were again unresponsive to GG immunization with the OVA vaccine, as indicated by the lack of IFN-γ secretion and their inability to kill SIINFEKL-pulsed target cells in vivo (Fig. 4B, 4C, left panel). In contrast, CTL activity against OVA was unaffected in LCre-GFP mice and comparable to that of Cre<sup>reg</sup> littermates (Fig. 3B, 3C, right panel). In a second approach, two groups of LCre-lacZ mice were treated with TAM to induce βGal expression in LCs. Then 4 wk later, mice were GG immunized with either βGal- or OVA-encoding DNA vaccines and analyzed for CTL functions against the respective Ags (Fig. 4D). Whereas LCre-lacZ mice were completely resistant to a subsequent challenge with the βGal DNA vaccine, they responded to OVA DNA with a strong CTL activation that was comparable to Cre<sup>reg</sup> controls (Fig. 4E, 4F, right panel). These data clearly indicate that LC-mediated CTL tolerance is exclusively directed toward the LC-borne neoantigen, without affecting CTL responses in general.
LC-mediated CTL tolerance relies on Tregs

LCs have been implicated in the suppression of immune reactions by activation or expansion of Tregs (18, 21, 43). Interestingly, at the site of GG challenge, we found Foxp3+ Tregs substantially increased in the skin of TAM-treated (tolerized) LCre-GFP-OVA mice but not in the Cre neg littermates (Fig. 5A). Therefore, we hypothesized that CTL tolerance might be due to Treg induction and/or local expansion. If so, Treg depletion before Ag challenge should restore CTL responses in tolerized mice to levels of GG-immunized Cre neg controls. Then 4 wk post-TAM treatment mice were GG immunized with either pCI-Ova or pCI-βGal (D) twice, at a 2 wk interval. Then 2 wk after the boost, mice were analyzed for specific lysis of SIINFEKL-pulsed target cells in vivo and IFN-γ secretion by CD8+ T cells after in vitro restimulation of spleen cells with SIINFEKL. Data show mean ± SD of groups of four to five individually analyzed mice and are representative of three independent experiments.

Activated LCs promote the development of a CTL memory response

Finally, we wished to investigate whether and how maturation stimuli would affect the tolerogenic functions of LCs. To this end, systemic TAM treatment of LCre-GFP-OVA mice was combined with intradermal injection of anti-CD40/poly(I:C) (22, 44). After 4 wk, mice were GG immunized with OVA plasmid and boosted after another 2 wk. After two additional weeks, cytotoxic T cell responses were analyzed in the SDLN and spleen of the respective mice (Fig. 6A). Contrary to the robust CTL tolerance induced by nonactivated LCs, priming of OVA-specific CD8+ T cells by activated LCs resulted in a strong activation of cytotoxic T cells. Moreover, the level of specific target cell killing (Fig. 6B, 6C) and...
FIGURE 5. Tregs are involved in LC-mediated CTL tolerance. (A) Then 4 wk after TAM treatment, LCre-GFPOVA and Cre<sup>−/−</sup> littermates were GG immunized twice at a 2 wk interval. Two weeks after the GG boost, the two spots of immunized skin and, as a control, skin from naive mice was isolated. Skin cells were gated on live CD3<sup>+</sup> CD4<sup>+</sup> lymphocytes and analyzed for CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs, as depicted on representative contour plots. Tregs are shown as % of CD4<sup>+</sup> T cells (left panel) and as cells per square centimeter of skin (right panel). (B) Groups of LCre-GFPOVA mice were treated with 2 mg TAM i.p. After 4 wk, mice received 250 μg of anti-CD25 Ab i.p. or were left without treatment. Next day, mice were GG immunized with pCI-Ova. Cre<sup>−/−</sup> littermates without anti-CD25 treatment served as WT controls (gray bar/symbols). Anti-CD25 treatment and GG immunization was repeated after 1 wk and then 1 wk later mice were analyzed for specific lysis of SIINFEKL-pulsed target cells in vivo. (C) LCre-GFPOVA mice were treated with 2 mg TAM. After 4 wk CD4<sup>+</sup>CD25<sup>+</sup> T cells were isolated from these mice and injected into naive BL6 recipients (3 × 10<sup>5</sup> T<sub>reg</sub>h) CD4<sup>+</sup>CD25<sup>+</sup> T cells, isolated from naive BL6 WT donors injected into BL6 mice served controls (white bars/symbols). Next day, recipient mice were GG immunized with pCI-Ova and boosted after 2 wk. Then 2 wks after the boost, the frequency of IFN-γ-producing CTL against OVA (Fig. 6D, 6E) was even higher as compared with Cre<sup>−/−</sup> control mice, suggesting the generation of recallable CD8<sup>+</sup> memory T cells.

Two hallmarks of long-lived CD8<sup>+</sup> central memory T cells are the expression of CD62L for LN homing and expression of the IL-7 receptor (IL-7R/CD127), required for T cell survival (45). To strengthen the above findings on a molecular level, we followed the expression kinetics of these two markers on endogenous OVA-specific CD8<sup>+</sup> T cells in LCre-GFPOVA mice. Strikingly, at all time points (days 9, 19, and 29) post-TAM treatment, the expression of CD127 and CD62L on Kb/SIINFEKL<sup>+</sup> CD8<sup>+</sup> T cells was weak in tolerized LCre-GFPOVA mice (Fig. 6F, left panels). However, both markers were strongly upregulated on CTL that had been primed by matured LCs (Fig. 6F, right panels). Together, these data show that presentation of OVA by activated LCs prevents CTL tolerance and directs cytotoxic T cells toward a recallable memory population.

Discussion

The presence of multiple DC subsets in skin, with partially distinct and partially overlapping features, complicates the functional analysis of LC in vivo. Genetic mouse models including those for the selective ablation of langerin<sup>+</sup> DC have allowed at least indirect conclusions to be drawn from the absence of these subsets, and added much to our current knowledge. Our study aimed to investigate the role of LCs in the orchestration of CTL responses under the condition that all DC subsets are in place and functional. To this end, we employed CreERT<sub>2</sub>/loxP-based mouse models for the inducible expression of Ags exclusively in LCs. Intracellular de novo synthesis of nonself-proteins is a hallmark of viral infection. Therefore, this approach could also be useful to model important aspects of immune reactions against viruses that infect LC, such as HIV (46).

In our inducible mouse model, de novo expression of GFPOVA in LCs transiently activated cytotoxic T cell responses against OVA. This was surprising, because CTL activation occurred in the absence of inflammatory signals. However, because TAM was administered systemically, Ag expression was also induced in the LCs of SDLN where steady-state LCs express higher levels of maturation markers than in the epidermis (47). In a previous study, increased expression of maturation markers was also found on bone marrow–derived DCs after selective disrupting E-cadherin-mediated DC-DC interactions (48). A similar scenario is conceivable in our model. The loss of E-cadherin interactions with epithelial cells could have resulted in steady-state maturation of LCs, which in turn transiently activated CTL responses. However, CTL that were activated by these LCs differed from such induced by inflammatory signals in two important aspects: they did not upregulate memory markers CD127 and CD62L, and they were unable to generate long-term CTL memory. It is still unclear whether this phenotype was shaped by inappropriate activation of CD8<sup>+</sup> T cells by steady-state LCs, or indirectly by the action of concomitantly induced T<sub>reg</sub>.

CTL induced by steady-state LCs seemed to go through a differentiation process that affects their responsiveness to APC: in the early phase of activation these CTL were sensitive to Ag challenge but became refractory at later time points. In fact, after resolution of
the primary response, animals remained resistant to subsequent GG immunization, indicating the development of immunological tolerance. Tolerance did not develop when TAM was combined with systemic anti-CD40/poly(I:C) treatment. Under these conditions, Ag expression elicited CD8+ T cells with upregulated memory markers. Importantly, such mice responded more vigorously than naive mice to a subsequent GG challenge, indicating the development of a functional CTL memory population. Thus, in our model, LCs reacted in accordance with the classical DC paradigm, that is, tolerogenic when immature and immunogenic when matured (49).

LC-mediated tolerance has been explained by various mechanisms, including IL-10 release from LCs (19), induction of Tregs (18), or purging the pool of Ag-specific T cells by abortive activation (20). In our model, it is unlikely that Ag-specific CD8+ T cells have been completely eliminated, as the depletion of CD25+ Tregs in tolerized mice restored the responsiveness to subsequent GG vaccination. From this we conclude that: 1) Ag-specific CD8+ T cells must have been retained in the system; and 2) Tregs were critical for steady-state LC-mediated tolerance. Involvement of Tregs was also demonstrated by the transfer of CD25+ cells from tolerized mice to naive hosts. These cells sup-
pressed GG-induced CTL reactions in the recipient mice stronger than CD25+ cells from naïve donors, suggesting the development of Ag-specific Tregs by neoantigen expression in LCs. In addition to this merely indirect hint of LC-mediated Treg development, adoptively transferred OT-2 cells differentiated to Tregs in TAMB-treated LCre-GFPPOVA mice.

Until recently, it was uncertain how Tregs control CD8+ T cells. However, a very recent study shed light on the mode of function, showing that IL-2 consumption by Tregs suppress the activation and expansion of memory CTL (50). The induction of CD4+ CD25+ Tregs requires that the cytosolic neoantigen is presented on MHC-II, a process that relies on autophagy (51, 52). However, whether, or under which conditions, steady-state LCs are capable of guiding cytoplasmic Ags into this pathway is currently unknown.

Although the principal capability of LCs to activate T cells is clearly beyond debate, good experimental evidence for the immunogenicity of LCs in vivo is surprisingly sparse and sometimes controversial (53–55). One reason for this is that the full complexity of skin DC subsets has only become evident in recent years (7, 56), and immunogenic functions ascribed to LCs in earlier papers may in fact have been carried out by other subsets (57, 58). More recently, in mice allowing for selective ablation of langerin+ DCs, LCs appeared to promote contact hypersensitivity reactions (59), but the opposite was found in very similar models and the reasons for that are still unclear (39, 60). A second example of the immunogenicity of LCs in vivo is their critical role for Th17 responses in Candida albicans infections (61).

In contrast, there is more literature documenting a tolerogenic role of LCs in vivo (54, 55), such as conditional MHC-II deficiency in LCs increased contact hypersensitivity responses (19). In a recent study, we found that cytotoxic and Ab responses were increased in LC-deficient mice immunized with OVA- but not βGal-encoding GG vaccines, suggesting that Ag-intrinsic features may influence the way they are handled by LCs (43). Notably, some papers demonstrated the suppressive effects of LCs on CD4+ or CD8+ T cell responses even in the context of strong maturation signals, suggesting that tolerogenicity could be an inherent feature of LCs that is also efficacious in (or even requires) inflammatory environments (12, 20, 22). These data show that bone marrow chimeras for MHC-II(-/-)-restricted Ag presentation exclusively by LC tolerated adoptively transferred Ag-specific CD4+ T cells, even though mice had been immunized with strong adjuvants (20). Ag conjugated to anti-langerin Abs was used to target LCs (but no other langerin+ DCs), and this resulted in CTL tolerance when administered with anti-CD40/poly(I:C) (22).

This is in clear conflict with our finding that systemic administration of anti-CD40/poly(I:C) prohibited the tolerogenic effect of LCs and reverted the response into protective immunity with recallable memory. A reason for these discrepancies between our model and those of Flacher et al. (22) may be some fundamental differences in experimental settings, such as: 1) direct presentation of endogenous Ags on MHC-I versus receptor-mediated uptake and cross-priming of exogenous OVA; 2) higher dose of CD40/poly(I:C) in the priming phase of CTL; and 3) Ag challenge (two GG challenges with the OVA plasmid versus one single injection of OVA protein). In support of our findings, inductive expression of a viral Ag in all CD11c+ DC has been shown to elicit tolerance in the absence, and protective immunity in the presence, of inflammatory signals (14, 62). If anything, it is conspicuous that studies based on some form of exogenous (i.e., not LC-born) Ag identify tolerogenicity as an invariable feature of LCs (12, 20, 22). In models employing DC-intrinsic Ags, inflammatory signals reverted tolerogenicity to immunogenicity (14, 62), a feature that might be evolutionary beneficial in settings of viral infection or LC cancer (e.g., LC histiocytosis).

In conclusion, our transgenic mouse models allow for the analysis of immune functions of LCs in vivo without any alteration of the complex composition of skin DC subsets. Our study provides evidence that steady-state LCs induce tolerance to LC-borne neoantigens, and that inflammation abrogates the tolerogenicity of LCs.

Disclosures
The authors have no financial conflicts of interest.

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