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Cross-presenting Langerhans cells are required for the early reactivation of resident CD8$^+$ memory T cells in the epidermis

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Tissue-resident memory CD8$^+$ T cells (T_{RM}) reside at sites of previous infection, providing protection against reinfection with the same pathogen. In the skin, T_{RM} patrol the epidermis, where keratinocytes are the entry site for many viral infections. Epidermal T_{RM} react rapidly to cognate antigen encounter with the secretion of cytokines and differentiation into cytotoxic effector cells, constituting a first line of defense against skin reinfection. Despite the important protective role of skin T_{RM}, it has remained unclear, whether their reactivation requires a professional antigen-presenting cell (APC). We show here, using a model system that allows antigen targeting selectively to keratinocytes in a defined area of the skin, that limited antigen expression by keratinocytes results in rapid, antigen-specific reactivation of skin T_{RM}. Our data identify epidermal Langerhans cells that cross-present keratinocyte-derived antigens, as the professional APC indispensable for the early reactivation of T_{RM} in the epidermal layer of the skin.

Langerhans cells | resident memory T cells | skin immunity | immunological memory | CD8$^+$ T cells

Tissue-resident memory CD8$^+$ T cells (T_{RM}) are a noncirculating subset of CD8$^+$ memory T cells that reside at the site of a previous infection. T_{RM} contribute to local immune surveillance by constantly patrolling through the tissue and scanning for cognate antigen (1, 2). After sensing cognate antigen, reactivated T_{RM} remain localized in the tissue, proliferate in situ (3, 4), differentiate into cytotoxic effector T cells (5), and secrete massive amounts of Interferon-γ (IFNγ), which induces a tissue-wide anti-viral state. (6). T_{RM} are of particular importance at epithelial surfaces such as the skin, where they provide protection against reinfection or reactivation of latent viruses, such as herpesviruses (7, 8). However, reactivation of T_{RM} has also been shown to contribute to autoimmunity that causes reoccurring patchy skin diseases like vitiligo, psoriasis, and cutaneous Lupus erythematosus (9). Thus, detailed knowledge of the cellular prerequisites for T_{RM} reactivation in the skin is important for understanding recurrent viral infections and inflammatory skin diseases.

It has been accepted for some time that both, priming of cytotoxic T cell responses against infectious agents (10, 11) as well as restimulation of central memory CD8$^+$ T cells requires dendritic cells (DC) as professional antigen-presenting cells (APC) and costimulation through CD28 (12). In contrast, the requirements to reactivate effector memory T cells or to trigger cytolytic function and cytokine secretion by differentiated cytotoxic T lymphocytes, both of which lack CD62L expression and are thus largely excluded from secondary lymphoid organs, are far less demanding and are thought to not require professional APC (13). As T_{RM} share properties with effector cells, such as expression of CD69 and a lack of CD62L, it appeared conceivable that their reactivation might not require professional APC. Indeed, recently published work clearly demonstrated that the reactivation of lung T_{RM} in the context of the Influenza virus did not require DC and could be mediated by both APC of hematopoietic and nonhematopoietic origin (14).

In the skin, T_{RM} reside in the epidermis, in the area of a previous viral infection (2, 6). In the steady state, Langerhans cells (LC) are the only professional APC that are present in the epidermal layer of the skin. Thus, a role of LC in the reactivation of skin resident memory has been deemed an attractive concept (15), but so far direct evidence for such a role has been elusive. On the other hand, many viruses that infect the skin replicate in keratinocytes (16). Therefore, major histocompatibility complex (MHC)-class I molecules on the keratinocyte surface will most likely be the first site where viral antigens become visible to T_{RM} in the skin. In order to shed light on the early events leading to functional reactivation of T_{RM} in the skin, we aimed to determine whether antigen presentation by keratinocytes alone is sufficient to induce local memory reactivation or whether professional APC are required and, if the latter is the case, which type of professional APC can fulfil this function.

Significance

We demonstrate here, using a genetic model that allows the inducible expression of a limited amount of antigen in a defined area of the epidermis that a professional antigen-presenting cell (APC) is required for the reactivation of skin resident CD8$^+$ memory T cells (T_{RM}) in vivo. Our data identify Langerhans cells as nonredundant cross-presenting APC essential for the early reactivation of epidermal T_{RM}. Our work sheds light on the early events after the reintroduction of antigen to a formerly infected skin area, thus contributing to our understanding of the processes mediating immune protection by skin resident memory T cells.

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To address this question, we made use of an inducible transgenic mouse model that targets the expression of a model-antigen to keratinocytes without changing the cellular composition of the steady-state epidermis (17). We used inducible antigen expression in keratinocytes in combination with mouse models, where certain types of APC are missing or can't present antigen, to investigate the prerequisites for T_{RM} reactivation. Our findings demonstrate that early functional reactivation of skin T_{RM} in response to antigens expressed in keratinocytes requires LC as professional cross-presenting APC.

Results

Antigen Expression by Keratinocytes Reactivates Skin T_{RM}. Skin resident memory $CD8^+$ T cells (T_{RM}) reside in the epidermal layer of the skin at the site of a previous infection where they rapidly respond to antigen reencounter with the secretion of inflammatory cytokines, including IFNγ, which can directly induce expression of antiviral genes in the surrounding tissue but also recruit and activate other immune cells (18). To investigate the cellular requirements for T_{RM} reactivation in the epidermis, i.e., whether reactivation requires a professional APC, we made use of a transgenic mouse model (K14DIETER), in which the expression of a model antigen containing the H2-D^b restricted epitope GP_{33-41}, derived from the lymphocytic choriomeningitis virus (LCMV) glycoprotein, can be induced selectively in keratinocytes through topical treatment with 4-hydroxy-tamoxifen (4OHT) (Fig. 1*A*) (17, 19, 20). GP_{33-41}-specific T cell receptor (TCR) transgenic $CD8^+$ T cells fromTCR327 x IFNγYFP ($CD90.1^+$), allowing for the direct readout of IFNγ production by yellow fluorescent protein (YFP) expression, were adoptively transferred into K14DIETER mice (Fig. 1*B*). Mice were subsequently infected on the ear pinnae with recombinant Vaccinia virus expressing the GP_{33-41} antigen (rVACV-G2) (Fig. 1*B*). Thirty days later, when the GP_{33-41} specific cells had differentiated to T_{RM} uniformly expressing CD69 and CD103 (Fig. 1*C* and *SI Appendix*, Fig. S1), antigen expression was induced by topical 4OHT application.

Antigen expression in keratinocytes resulted in functional reactivation of skin T_{RM}, as indicated by expression of the IFNγ-YFP reporter, as early as 24 h after 4OHT treatment. Importantly, early reactivation, 24 h and 36 h after antigen expression in keratinocytes, was exclusively found in T_{RM} isolated from the epidermis. Only after 48 h, some TCR327 × IFNγYFP cells, expressing the IFNγ-YFP reporter could be detected in the dermis (Fig. 1*D* and *SI Appendix*, Fig. S1). During the first 48 h after 4OHT treatment, the number of specific T_{RM} in the epidermis or dermis did not significantly change (Fig. 1*E*). However, antigen-specific reactivation of T_{RM} resulted in substantial ear swelling, which became apparent 24 h after induction of antigen expression (Fig. 1*F*). The cellular infiltrate in this phase consisted largely of $CD11b^+Ly6C^+$ monocytes (Fig. 1*G*) and $CD11b^+Ly6G^+$ granulocytes (Fig. 1*H*). Reactivation of T_{RM} resulted in robust expression of IFNγ-induced transcripts *Ifitm3* encoding for Interferon-induced transmembrane protein 3 and *Ip-10* encoding for C-X-C motif chemokine ligand (CXCL) 10 in the skin (Fig. 1*I*).

Taken together, these data show that expression of antigen selectively on keratinocytes is sufficient to rapidly reactivate antigen-specific skin resident memory $CD8^+$ T cells in the epidermis, resulting in IFNγ production, recruitment of monocytes and granulocytes and, through expression of IFNγ induced genes, establishment of an antiviral state in the skin.

Langerin$^+$ Cells Are Required for the Reactivation of Skin T_{RM} by Keratinocyte-Derived Antigens. Keratinocytes are the first site of replication for many viruses such as arboviruses, herpes

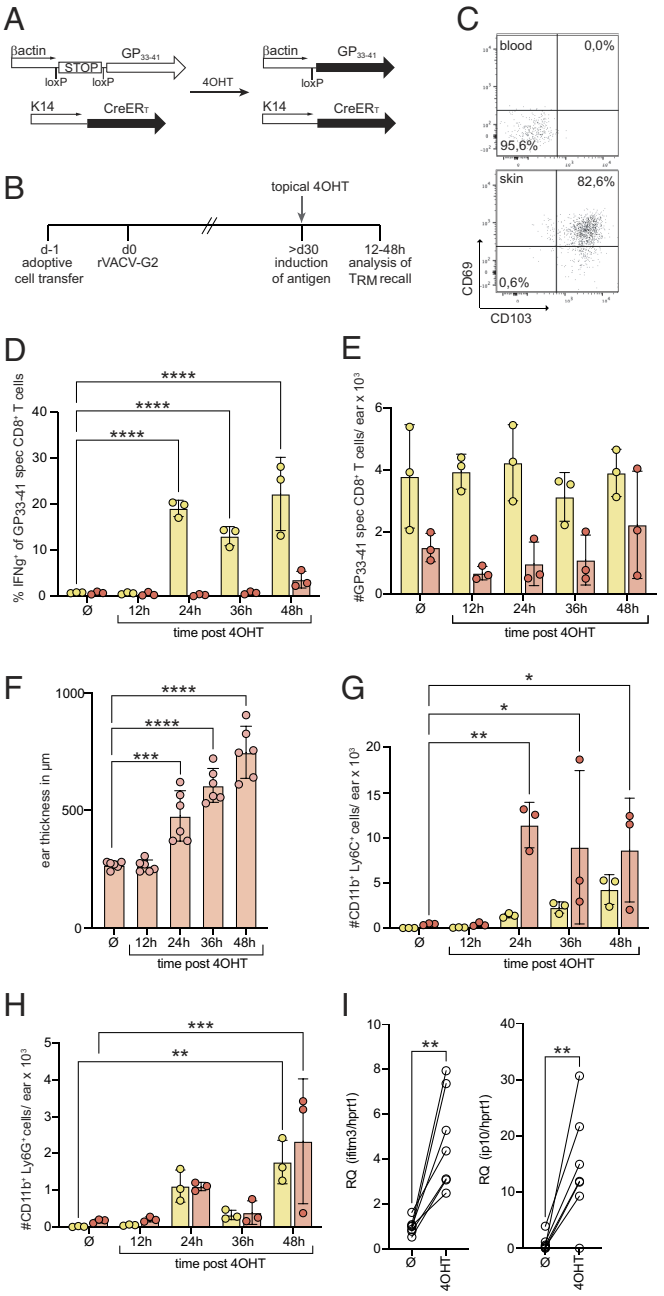

Fig. 1. Antigen expression in keratinocytes triggers rapid functional reactivation of T_{RM} in the epidermis. (*A*) K14DIETER mice express a tamoxifen inducible Cre recombinase ($CreER_T$) under control of the Cytokeratin14 promoter. Upon ligation of 4-hydroxytamoxifen (4OHT), Cre can excise a loxP-flanked STOP cassette, activating expression of the LCMV-derived H-$2D^b$-restricted CTL epitope GP_{33-41}. Thus, topical treatment of the skin with 4OHT induces expression of the peptide GP_{33-41} selectively in keratinocytes in the treated area of the skin. (*B*) Procedure for skin resident memory reactivation experiments. K14DIETER mice were adoptively transferred with IFNγYFP-TCR327 $CD8^+$ T cells ($CD90.1^+$). One day later, both ears were infected with recombinant Vaccinia virus expressing the LCMV Glycoprotein (rVACV-G2). 30d p.i. antigen expression was induced by topical 4OHT treatment on the ear. (*C*) Representative flow cytometry plots of $CD8^+CD90.1^+$ IFNγYFP TCR327 T cells in ear skin and blood 30 d after rVACV-G2 infection. (*D*) YFP expression in $CD8^+CD90.1^+$ IFNγYFP-TCR327 T cells and (*E*) absolute number of $CD8^+CD90.1^+$ IFNγ-YFP TCR327 T cells in Epidermis (yellow) and dermis (red). (*F*) Ear thickness at indicated times after 4OHT treatement. (*G* and *H*) Absolute numbers of $CD11b^+Ly6C^+$ cells (*G*) and $CD11b^+Ly6G^+$ cells (*H*) in epidermis (yellow symbols) and dermis (red symbols) per ear at indicated times after 4OHT treatment. (*I*) Expression of IFNγ stimulated genes in the skin of 4OHT treated (4OHT) or untreated (ø) ear skin 48 h after induction of antigen expression in keratinocytes is shown with n = 4 to 7 per group. One of three independent experiments is shown. Vertical bars represent mean ± SD. Statistical significance was determined using one-way ANOVA comparing each timepoint in either epidermis or dermis with the respective untreated control (flow cytometry) or paired *t* test (qPCR); with $^*P \le 0.1$; $^{**}P \le 0.01$ $^{***}P \le 0.001$; $^{****}P \le 0.0001$ all other comparisons not significant.

viruses, papillomaviruses, or vaccinia virus (16). Thus, direct recognition of viral antigens presented on MHC-class I molecules on keratinocytes would be the fastest way to reactivate T_{RM} in case of a viral reinfection, and indeed, direct antigen-presentation by human keratinocytes has been shown to reactivate memory $CD8^+$ T cells in vitro (21). Alternatively, reactivation of T_{RM} by antigens expressed in keratinocytes might require cross-presentation by a professional APC. To establish whether a cross-presenting APC is necessary, we bred K14DIETER mice to LangerinDTR mice (22) expressing a human diphtheria toxin (DT) receptor from the endogenous langerin locus, thus allowing for the depletion of both APC populations in the skin that have been shown to be capable of cross-presentation (23–25), $Langerin^+$ cDC1 and LC (23, 26, 27).

K14DIETER × LangerinDTR mice ($LangerinDTR^+$ and, as control, $LangerinDTR^-$ littermates) were adoptively transferred with $CD8^+$ T cells isolated from TCR327 × IFNγYFP or TCR327 × Nur77eGFP mice (both $CD90.1^+$) and infected with rVACV-G2. Thirty days later, when skin resident memory was established, mice were injected with DT to deplete $Langerin^+$ cells in $LangerinDTR^+$ mice but not in $LangerinDTR^-$ controls. Subsequently, antigen expression by keratinocytes was induced by topical application of 4OHT on one ear of each mouse. Treatment of mice with two doses of DT at day-3 and day-1 completely eliminated both $MHC\text{-}II^+CD11c^+XCR\text{-}1^+$cDC1 and $MHC\text{-}II^+CD11c^+EpCAM^+$ LC (Fig. 2 *A* and *B* and *SI Appendix*, Fig. S2). Induction of antigen expression in keratinocytes resulted in a strong reactivation of T_{RM} in undepleted mice, evidenced by ear swelling in 4OHT-treated versus untreated ears (Fig. 2*C*) and expression of the IFNγ reporter (Fig. 2*D*). This reactivation was completely abrogated in mice that had been depleted of $Langerin^+$ cDC1 and LC, suggesting that Langerin expressing professional APC are required for the reactivation of skin T_{RM} in response to an antigen expressed in keratinocytes. To determine whether impaired reactivation was caused by impaired TCR triggering in the absence of $Langerin^+$ cells or whether direct antigen recognition on keratinocytes could trigger the TCR of T_{RM} and $Langerin^+$ APC would provide a noncognate, auxiliary signal required for functional reactivation of T_{RM}, we made use of Nur77eGFP mice. In these mice, enhanced green fluorescent protein (eGFP) expression in T cells directly reflects TCR triggering independent of costimulation or cytokine sensing (28). Depletion of $Langerin^+$ cells also abrogated eGFP expression from TCR327 × Nur77eGFP ($CD90.1^+$) T_{RM} upon antigen induction (Fig. 2*E*), indicating that $Langerin^+$ cells are required as APC providing the cognate signal for T_{RM} reactivation in response to keratinocyte-expressed antigens.

Radioresistant Cells Mediate the Reactivation of T_{RM} in the Skin. We had observed early reactivation of T_{RM} in response to antigen expressed in keratinocytes exclusively in T_{RM} in the epidermis, suggesting that the $Langerin^+$ APCs responsible were LC, which

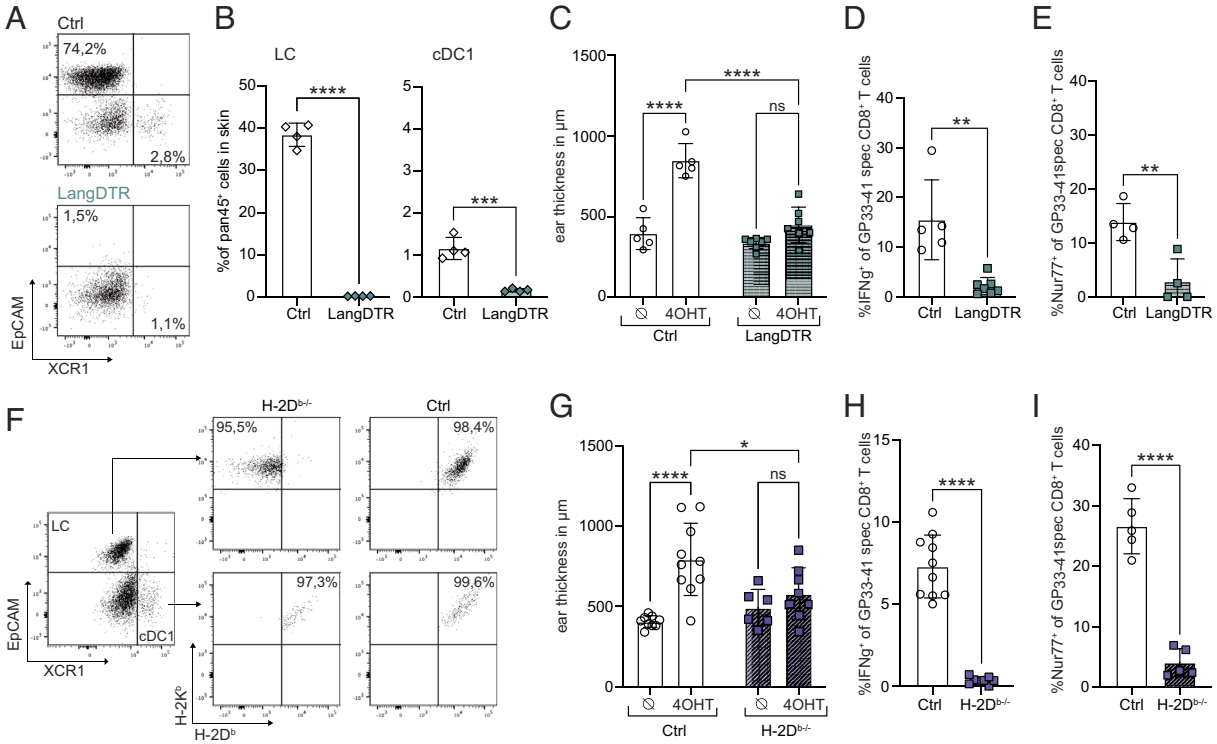

Fig. 2. $Langerin^+$ cells and antigen presentation on radioresistant cells are required for reactivation of T_{RM} in the skin. (*A–C*) K14DIETERxLangerinDTR mice were adoptively transferred with IFNγYFP- or Nur77eGFP- TCR327 $CD8^+$ T cells ($CD90.1^+$) and infected on the ears with rVACV-G2. Thirty days after infection, mice were injected with DT to deplete $Langerin^+$ cells in $LangerinDTR^+$ K14DIETER (LangDTR) but not in $LangerinDTR^-$ K14DIETER (Ctrl). (*A*) Representative flow cytometry plot of $CD11c^+$ $MHC\text{-}II^+$ cells from the ear 1 d after DT treatment and (*B*) cumulated data from four mice, showing depletion of $EpCAM^+$ LC and $XCR1^+$ cDC1. (*C*) Ear thickness of 4OHT treated (4OHT) or of untreated (∅) ears, and (*D* and *E*) reporter gene expression in IFNγYFP-TCR327 $CD8^+$ T cells (*D*) and Nur77eGFP-TCR327 $CD8^+$ T cells (*E*) in 4OHT-treated ears at 48 h after induction of antigen expression in keratinocytes. Symbols indicate individual mice of n = 4 to 7 per group. (*F–I*) Bone marrow chimeric mice were generated by transplanting lethally irradiated K14DIETER x H-2 $D^{b-/-}$ (H-2 $D^{b-/-}$) or K14DIETER (Ctrl) mice with WT bone marrow. After reconstitution, DC subsets in ear skin cell suspensions from chimeras were analyzed by flow cytometry. (*F*) Expression of H-2 K^b and H-2 D^b in $EpCAM^+$ LC and $XCR1^+$ cDC1 (pregated on $CD11c^+$ $MHC\text{-}II^+$ cells). Numbers indicate frequency of cells in the respective quadrant. (*G–I*) Chimeras were adoptively transferred with IFNγYFP- or Nur77eGFP-TCR327 $CD8^+$ T cells ($CD90.1^+$) and infected on the ears with rVACV-G2. Thirty days after infection, antigen expression in keratinocytes of one ear was induced by topical 4OHT treatment while the other ear was left untreated (∅). (*G*) Ear thickness of 4OHT-treated (4OHT) or of untreated (∅) ears and (*H* and *I*) reporter gene expression in IFNγYFP-TCR327 $CD8^+$ T cells (*H*) and Nur77eGFP-TCR327 $CD8^+$ T cells (*I*) in 4OHT-treated ears at 48 h after induction of antigen expression in keratinocytes. Symbols represent individual mice of n = 5 to 10 per group. Vertical bars represent mean ± SD. Statistical significance was determined with two-way ANOVA (*C* and *G*) or unpaired *t* test with $^*P \le 0.1$; $^{**}P \le 0.01$ $^{***}P \le 0.001$; $^{****}P \le 0.0001$; ns (not significant).

are the only professional APC located in the steady-state epidermis, rather than dermal Langerin$^+$ cDC1. To delineate whether the cell type responsible for T_{RM} reactivation by keratinocyte-derived antigens were dermal cDC1 or epidermal LC, we made use of the differential sensitivity to irradiation of these two cell types. LC are radioresistant and are not replaced by bone marrow–derived cells after lethal irradiation followed by bone marrow transplantation, whereas cDC1 are completely replaced by cells derived from the bone marrow transplant (24, 29). To generate a situation, where radioresistant cells were incapable of presenting the GP_{33-41} model antigen, we crossed K14DIETER mice to H-2D$^{b-/-}$ mice, which lack the restricting MHC class-I molecule. K14DIETERx H-2D$^{b-/-}$ mice were lethally irradiated and transplanted with bone marrow from wild-type mice. Eight weeks after transplantation, cDC1 were exclusively derived from the wild-type congenic transplant, whereas LC, in contrast to cDC1, remained of recipient origin and thus were deficient for H-2D^b (Fig. 2*F*). In these chimeric mice, expression of the model antigen can be induced by topical 4OHT treatment but LC and the keratinocytes themselves are deficient for H-2D^b and thus cannot present the GP_{33-41} epitope, whereas dermal DC express H-2D^b and can present the model antigen (Fig. 2*F*). As a control, K14DIETER mice were transplanted with wild-type bone marrow (Ctrl). Bone marrow chimeric mice were adoptively transferred with TCR327 × IFNγYFP cells or TCR327 × Nur77eGFP (CD90.1$^+$) and infected on the ears with rVACV-G2. Thirty days after infection, when skin resident memory was established, antigen expression in keratinocytes was induced by topical 4OHT treatment on one ear of each mouse. Forty-eight hours after antigen induction, ear thickness (Fig. 2*G*) and reactivation of T_{RM} was assessed by flow cytometric quantification of IFNγYFP or Nur77eGFP reporter expression (Fig. 2 *H* and *I* and *SI Appendix*, Fig. S1). As before, induction of antigen expression in keratinocytes led to a robust reactivation of T_{RM} in K14DIETER chimeric mice, where all cells are competent to present the GP_{33-41} epitope. This reactivation was completely abrogated in K14DIETER x H-2D$^{b-/-}$ chimera, where radioresistant cells lack the restricting MHC class-I molecule, indicating that antigen presentation on a radioresistant cell population is necessary for T_{RM} reactivation in the skin. Collectively, these data demonstrate that early reactivation of T_{RM} in the skin depends on Langerin$^+$ cells (Fig. 2 *A–E*), but not on radiosensitive cells like, e.g., Langerin$^+$ cDC1, and requires antigen presentation by radioresistant cells (Fig. 2 *F–I*), criteria are only fully met by LC.

Langerhans Cells Cross-Present Keratinocyte-Derived Antigen. In order to mediate reactivation of T_{RM} in the skin in response to antigen expression in keratinocytes, LC need to be able to: 1) take up antigen produced in keratinocytes, process it, and present it bound to MHC-class I molecules on their surface (cross-presentation) and 2) form physical contact with T_{RM} in the skin. To investigate the ability of LC to cross-present keratinocyte-derived antigen, we treated naive K14DIETER mice with 4OHT to induce expression of the model antigen on keratinocytes. Forty-eight hours after antigen induction, we isolated LC from epidermal sheets and purified them by density gradient centrifugation and flow cytometric sorting for CD45$^+$ MHC-II$^+$ EpCAM$^+$ cells. (Fig. 3*A*). Presentation of the GP_{33-41} on H-2D^b purified LC was assessed by the ability to induce GFP expression in naive TCR327xNur77eGFP CD8$^+$ T cells, indicative of TCR signaling through the D^b /GP_{33-41}-specific TCR (Fig. 3*A* and *SI Appendix*, Fig. S3*A*). We observed strong TCR stimulation of D^b/GP_{33-41}-specific T cells by purified LC. Using the same assay to detect GP_{33-41} presentation on keratinocytes (CD45$^-$) purified by flow cytometry from 4OHT treated K14DIETER epidermal skin, we found that direct presentation of GP_{33-41} on keratinocytes can trigger specific T cells in vitro, but only at considerably higher APC/T cell ratios (Fig. 3*B*). Taken together, this directly demonstrates cross-presentation of the keratinocyte-expressed model antigen by epidermal LC and is well in line with previous reports showing cross-presentation of other keratinocyte-produced antigens on LC in mice (23, 30). A strong cross-presenting activity has also been demonstrated for human LC, suggesting that human LC also have the capacity to reactivate skin T_{RM} in response to

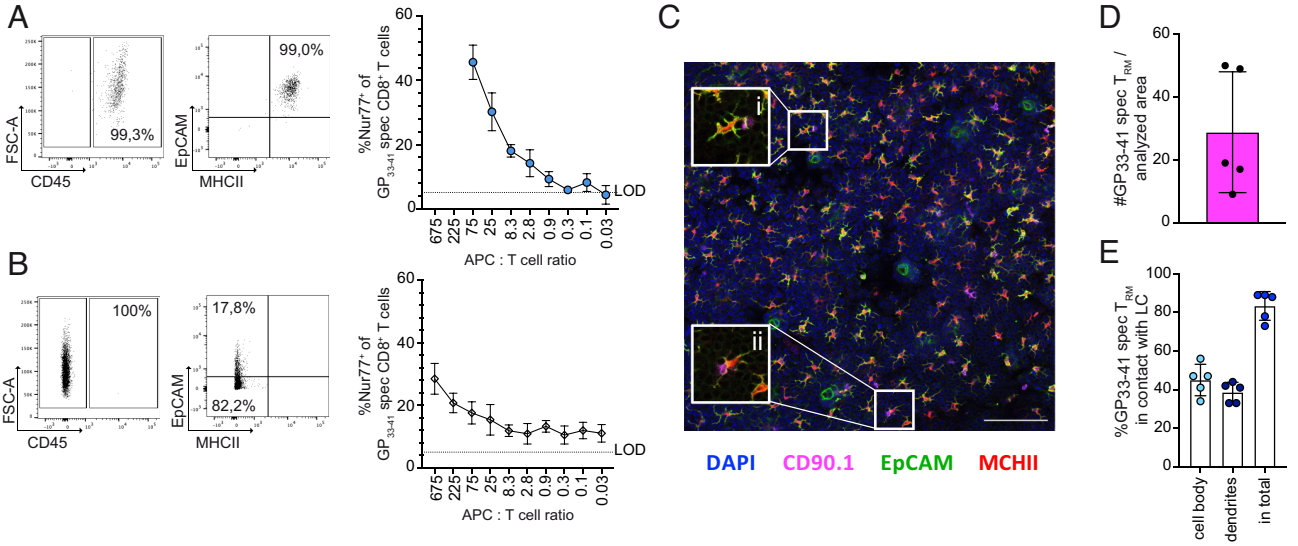

Fig. 3. LC present keratinocyte-derived antigen and are in contact with T_{RM} in the epidermis. (*A* and *B*) Naive K14DIETER mice were topically treated on the ear skin with 4OHT for antigen expression in keratinocytes. Two days later LC and keratinocytes devoid of any CD45$^+$ leukocyte contamination were isolated from the epidermis and purified by flow cytometric cell sorting. Purified LC (*A*) and keratinocytes (*B*) (representative flow cytometry plots after purification are shown) were cultured for 16 h in serial dilutions with naive Nur77eGFP-327TCR CD8$^+$ T cells followed by flow cytometric detection of GFP expression. n = 3 to 5. Detection limit (LOD) was determined using unstimulated CD90.1$^+$ Nur77eGFP-327TCR CD8$^+$ T cells. (*C*) Fluorescence microscopy of epidermal sheets of K14DIETER mice adoptively transferred with TCR327 CD8$^+$ T cells (CD90.1$^+$) and infected with rVACV-G2 30 d previously, stained for MHC-II (red), EpCAM (green), CD90.1 (magenta), and nuclei with DAPI (blue). Objective 20× 0.75 NA. (Scale bar: 100 μm.) Zoomed-in areas exemplarily show contacts between a T_{RM} and an LC's dendrites (i) or its body (ii) (*D*) Number of CD90.1$^+$ TCR327 T cells per imaged area of five individual epidermal sheets. (*E*) Frequency of CD90.1$^+$ TCR327 T cells in (*C*) in contact with the cell body or with dendrites of MHC-II$^+$ EpCAM$^+$ LC.

antigens expressed in keratinocytes (26, 31, 32). Of note is that the presentation of keratinocyte-derived antigens on LC might also involve the recently described mechanism of mRNA transfer from keratinocytes to LC followed by translation in LC (33).

LCs, which represent 3 to 5% of all nucleated cells in the epidermis, are located in the *stratum spinosum* of the epidermis, where they form a dense network spanning the whole body surface (34). Dendrites of LC reach between keratinocytes but also penetrate tight junctions to extend into the *stratum corneum* (35), allowing the LC to sample both antigens expressed in keratinocytes as well as antigens on the surface of the skin. In contrast to LC, $CD8^+$ T_{RM} are not as densely packed in the skin tissue but show a steady-state crawling behavior in between keratinocytes, searching the surrounding area for cells presenting their cognate antigen (1, 2). To visualize potential physical contact between rVACV-G2-primed GP_{33-41}-specific T_{RM} and LC in the epidermis, we analyzed epidermal sheets 30 d after infection by confocal microscopy. We found that indeed a substantial proportion of GP_{33-41} T_{RM} in the epidermis were in direct contact with an LC, either with the cell body or the dendrites of the LC (Fig. 3 *C–E*). In conclusion, LC have the ability to acquire keratinocyte-derived antigen and cross-present it on MHC class-I molecules to epidermal T_{RM}, with which they make frequent contact in the steady state. Recently, a population of Langerin$^+$ EpCAM$^+$ cells has been described in the dermis that is long-lived and radioresistant but is gradually being replenished with cells derived from bone marrow. These cells, termed LC^{like} cells, have the capacity to migrate to the draining lymph node and might have previously been mistakenly identified as LC in transit (36). However, our data showing that early reactivation of T_{RM} is restricted to the epidermis strongly suggest that epidermal LC rather than these dermal LC^{like} cells are responsible for early T_{RM} reactivation.

LCs Are Required for Reactivation of T_{RM} in the Skin Irrespective of Innate Immune Activation. Our data thus far show that in the absence of additional innate immune activation, reactivation of epidermal T_{RM} by limited antigen expression in keratinocytes requires cross-presenting LC as APC. To determine, whether the presence of innate immune activation at the time of antigen reintroduction would influence T_{RM} reactivation and its dependence on LC, we sought to apply a pathogen receptor ligand at the time of antigen reintroduction. To isolate the role of LC from a role of cDC1 in this experiment, we made use of the different repopulation kinetics of cDC1 and LC after depletion (37). K14DIETER x LangerinDTR mice were adoptively transferred with TCR327 × IFNγYFP cells or TCR327 × Nur77eGFP (CD90.1$^+$) and infected on the ears with rVACV-G2. Thirty days after infection, when skin resident memory was established, mice were treated twice with DT to deplete Langerin$^+$ cells or left untreated. Twelve days after the second DT treatment, when the XCR1$^+$ dermal cDC1 compartment was fully reconstituted in DT-treated mice but EpCAM$^+$ LC were still absent (Fig. 4 *A* and *B*), expression of antigen in keratinocytes was induced. To provide an innate immune activating stimulus at the time of antigen reintroduction, some mice were topically treated with an emulsion containing the toll-like receptor 7 (TLR7) agonist Imiquimod (IMQ), concomitantly with antigen induction. We have previously demonstrated that topical IMQ treatment strongly activates innate immune cascades through the TLR7/MyD88 pathway (38). Analysis of T_{RM} reactivation by assessing ear swelling (Fig. 4*C*), expression of the IFNγYFP reporter (Fig. 4*D*), and TCR stimulation reflected by Nur77eGFP expression (Fig. 4*E*) 24 h after 4OHT treatment revealed that antigen reintroduction in the

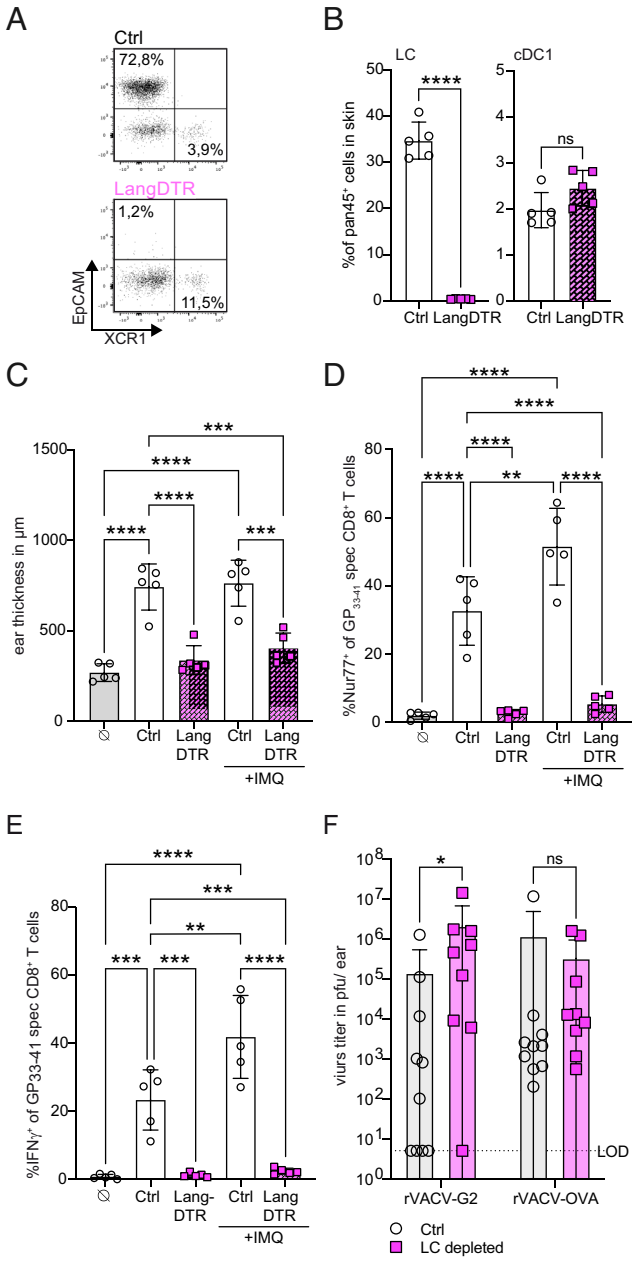

Fig. 4. Reactivation of skin T_{RM} and protection against viral challenge are dependent on LCs. (*A–E*) K14DIETERxLangerinDTR mice were adoptively transferred with IFNγYFP- or Nur77eGF-TCR327 $CD8^+$ T cells (CD90.1$^+$) and infected on the ears with rVACV-G2. Thirty days after infection, mice were injected with DT to deplete Langerin$^+$ cells in LangerinDTR$^+$ K14DIETER (LangDTR) but not in K14DIETER (Ctrl). (*A*) Representative flow cytometry plots and (*B*) cumulative data of CD11c$^+$ MHC-II$^+$ cells from the ear 14 d after DT treatment, showing the absence of EpCAM$^+$ LC but repopulation with XCR1$^+$ cDC1. (*C–E*) Fourteen days after DT treatment, antigen expression in keratinocytes was induced by topical 4OHT treatment alone or with additional Imiquimod (IMQ) on the ear skin of LC depleted and Ctrl mice. Twenty-four hours later, tissue thickness of 4OHT-treated (4OHT) or untreated (∅) ears (*C*) and Nur77eGFP (*D*) and IFNγYFP (*E*) reporter expression in specific T_{RM} quantified. n = 5 per group. Vertical bars represent mean ± SD. Statistical significance was determined with one-way ANOVA or unpaired *t* test (Fig. 4*B*) with $^*P \le 0.1$; $^{**}P \le 0.01$ $^{***}P \le 0.001$; $^{****}P \le 0.0001$; ns (not significant). (*F*) Wild-type and LangerinDTR mice were transcutaneously immunized against the GP_{33-41} antigen. Thirty days later, mice were injected with DT to deplete Langerin$^+$ cells in LangerinDTR (LC depleted, pink symbols) but not in wild-type mice (Ctrl, white symbols). Fourteen days later, when LC were still depleted in LangerinDTR mice but cDC1 had repopulated the skin, mice were infected on one ear with rVACV-G2 expressing the GP_{33-41} antigen and at the other ear with a control virus (rVACV-OVA) lacking GP_{33-41}. Virus titer in skin tissue was determined 4 d after infection for each ear. One experiment with n = 9 to 10 per group is shown. Vertical bars represent mean ± SD. Statistical significance was determined with nonparametric *t* test with $^*P \le 0.1$; $^{**}P \le 0.01$ $^{***}P \le 0.001$; $^{****}P \le 0.0001$; ns (not significant).

presence of innate immune activation induced TCR signaling and IFNγ production in a significantly higher proportion of T_{RM} as compared to reintroduction in the absence of an additional innate stimulus. Nevertheless, even in the presence of innate activation, T_{RM} reactivation was fully abrogated in LC-depleted mice, despite the presence of Langerin$^+$ XCR1$^+$ cDC1. Taken together, these data show that reactivation of T_{RM} by antigen expressed in keratinocytes depends on LC irrespective of innate immune activation.

Finally, we asked whether protection conferred by epidermal T_{RM} against a viral infection of the skin was dependent on the presence of LC. As we aimed to isolate protection conferred by CD8$^+$ T_{RM} from other aspects of adaptive immunity that might contribute to control of a viral reinfection, such as CD4$^+$ T cell and antibody responses, we sought to induce epidermal T_{RM} against a single MHC-I restricted epitope by an alternative method. We had previously established transcutaneous peptide immunization, where topical application of an antigenic peptide in an adjuvant-containing cream induces robust cytotoxic T cell responses and skin T_{RM} (39). We transcutaneously immunized LangerinDTR and control C57BL/6 mice on both ears with the GP_{33-41} peptide. Thirty days later mice were injected with DT to deplete Langerin$^+$ cells in LangerinDTR but not control mice. Fourteen days later, when Langerin$^+$ cDC1 had repopulated the skin but LC were still absent, mice were infected on one ear with recombinant Vaccinia Virus expressing the GP_{33-41} antigen (rVACV-G2) and on the other ear with a virus expression an irrelevant control antigen. Virus titers in the ears were determined 4 d later, at which time virus titers in nonimmune animals reach their maximum. We observed a 10-fold reduction in virus titers for the virus carrying the GP_{33-41} antigen as compared to the irrelevant control virus in mice with an intact LC compartment. (Fig. 4*F*). This protection was lost entirely in mice lacking LC, indicating that LC are critical for antiviral protection conferred by T_{RM} in skin.

Collectively, our data unequivocally demonstrate an essential and nonredundant role of LC as the APC, that induce the rapid functional restimulation of CD8$^+$ T_{RM} cells in the skin both in the presence and absence of innate immune stimulation.

Previous work has uncovered the respective roles of keratinocytes and LC for the retention of T_{RM} in the epidermis, demonstrating that T_{RM} retention requires autocrine secretion of TGFβ by T_{RM} and its activation by keratinocyte-expressed integrins (40, 41). While LC were found required for T_{RM} retention in a graft versus host disease model (42), they were found dispensable for the retention of virus-primed T cells in the epidermis (43, 44).

In our study here, we used an inducible antigen transgenic mouse model, in which topical application induces antigen expression in a limited number of keratinocytes within a defined skin area. Under these circumstances of limited antigen expression, we show that although antigen-expressing keratinocytes can stimulate T cells in vitro, they fail to induce TCR signaling in T_{RM} in vivo. This is somewhat in contrast to a study using constitutive antigen expression in all keratinocytes and intradermal injection of naive TCR transgenic T cells, which suggested keratinocytes can directly activate T cells, when antigen is expressed at high levels (45). Also, while some studies using adoptive transfer of naive T cells into mice expressing antigen in keratinocytes found antigen presentation by a radioresistant cell population involved in T cell activation (46), others identified bone marrow–derived CD207$^+$ cDC1 as essential APC population for T cell stimulation (24). The latter is in line with an earlier study showing that rejection of a skin graft transgenically expressing ovalbumin in keratinocytes requires cross-presentation by a bone marrow–derived APC (47). Thus, the requirement of a professional APC for T cell stimulation by keratinocyte-derived antigens might depend on the number of cells expressing the antigen and on antigen expression levels. Our finding that LC are required for the protection that T_{RM} confer against a challenge infection of the skin with vaccinia virus suggests that at antigen loads reached, at least in this infectious situation, LC as a professional APC are required for functional T cell receptor triggering.

Collectively, our data show that LC, which are, in the absence of prior inflammation and immune infiltration, the only mononuclear phagocyte population in the epidermis, have a nonredundant role as professional APC for T_{RM} reactivation. These results suggest that reactivation of T_{RM} by LC might be of particular importance for the protection against secondary infection by viruses that exclusively replicate in squamous epithelial cells, such as papillomaviruses (48). Should a pathogen trigger innate immune activation, at some point, other types of mononuclear phagocytes may infiltrate the epidermis and possibly be capable of cross-presenting antigen to T_{RM}. However, our data suggest that even when innate immunity is triggered through pathogen recognition receptors, the early reactivation of T_{RM} is entirely dependent on LC. Importantly, viruses for which keratinocytes are the primary host cell upon reinfection or reactivation from latency might evade pathogen recognition in keratinocytes and thus impair innate immune mechanisms. In particular, large DNA viruses such as papillomaviruses, poxviruses, and herpesviruses employ a plethora of mechanisms that block sensing of virus through cytosolic pattern recognition receptors (49–52). Rapid induction of IFNγ secretion by T_{RM} through cross-presentation by a professional APC which itself remains uninfected is an efficient way to overcome the blockade of cytosolic pathogen receptor signaling and Type I interferon production in infected cells. Thus, the early reactivation of T_{RM}, which through the secretion of IFNγ induce the expression of antiviral genes in the tissue but also recruit and activate other immune cells, might be in many cases the earliest trigger of an efficient immune response to reinfection of the epidermis.

Conclusion

Using a model system that allows antigen targeting selectively to keratinocytes in a defined area of the skin, we show that antigen expression in keratinocytes results in rapid, antigen-specific reactivation of skin T_{RM}. Our data identify epidermal LC as the nonredundant APC type for early CD8$^+$ T_{RM} reactivation in the epidermis. The reactivation of T_{RM} has been shown to rapidly trigger innate and adaptive immune mechanism that protect against tissue reinfection or reactivation of latent viruses (6, 53, 54). Our results suggest that in the skin, where keratinocytes are the target cell for many virus infections, cross-presentation of keratinocyte-expressed viral antigens on LC is essential for the protective function of T_{RM} against recurrent infection or reactivation from latency of skin tropic viruses. On the other hand, the presentation of autoantigens by LC and aberrant reactivation of T_{RM} might contribute to autoimmune skin diseases (9).

Materials and Methods

Mice. All mice were bred and kept under specific pathogen-free conditions at the Translational Animal Research Center of the University of Mainz, Mainz, Germany, and were on a C57BL/6 J background. Experiments were conducted following institutional guidelines and with permission of the State of Rhineland Palatinate.

K14DIETER double-transgenic mice Tg(KRT14-cre/ERT)20Efu/J × Tg(ACTB-GP33/NP396/bGal497/EGFP)69Gkl) allow the tamoxifen-inducible presentation of three LCMV-derived CTL epitopes (GP_{33-41}/Db, GP_{34-41}/Kb, and $NP_{396-404}$/Db) and one β-galactosidase-derived CTL epitope (β-$Gal_{497-504}$/Kb) by Cytokeratin 14 expressing cells (keratinocytes).

IFNγYFP reporter mice (B6.129S4-Ifng$^{tm3.1Lky}$/J) hold the "interferon-gamma reporter with endogenous polyA transcript" (GREAT) allele, which allows the identification of IFNγ producing cells based on eYFP expression.

TCR327 (B6;D2-Tg(TcrLCMV)327Sdz/J) mice carry the P14 transgenic T cell receptor that recognizes the LCMV-gp-derived epitope GP_{33-41} in the context of H-2D^b (55) and were bred to B6.Thy1.1 congenic mice.

Nur77eGFP (C57BL/6-Tg(Nr4a1-EGFP/cre)820Khog/J) reporter mice express eGFP under control of the Nr4a1 (Nur77) promoter (28).

H-2D$^{b-/-}$ mice (B6.129P2-H2-Db^{tm1Bpe}) lack a functional gene for the MHC-class I molecule *H-2D^b* (56). K14DIETER mice were bred to H-2D$^{b-/-}$ mice to create a mouse model where GP_{33-41} peptide presentation is disabled due to the lack of the presenting MHC class I molecule.

LangerinDTR (B6.Cd207tm1(DTR/EGFP)Bjec) mice harbor a targeted insertion of the human Diphteria toxin receptor (DTR) fused to eGFP in the Langerin locus and allow the depletion of Langerin expressing cells by diphtheria toxin (DT) injection (22).

B6.PL-Thy1a/CyJ mice carry the congenic marker CD90.1 on all T cells, respectively, and allow identification of transferred cells by flow cytometry.

Generation of Bone Marrow Chimeric Mice. Bone marrow was isolated by flushing the tibiae and femora of donor mice with Hanks' Balanced Salt Solution. Then, 5×10^6 bone marrow cells were injected i.v. into mice that had been lethally (9.5 Gy) irradiated using a ^{137}Cs source. Each mouse received daily circa 1 mg sulfadoxin and 0.2 mg trimethoprim (BORGAL - Bayer) via the drinking water for 3 wk and were rested 8 wk after transplantation before use in experiments.

Adoptive Transfer of TCR327 CD8$^+$ T Cells. Splenocytes were isolated from the spleen by smashing the organ through a 70-μm cell strainer. Then, 1×10^5 splenocytes in 100 μL HBBS were injected i.v. into recipient mice 1 d before viral infection.

Vaccinia Infection. Recombinant vaccinia virus expressing the LCMV-WE glycoprotein 2 (rVACV-G2) (57) was propagated on BSC-40 cells at low M.O.I. For skin infection, mice were anaesthetized i.p. with Ketamine/Xylazine. Then, 2×10^6 pfu of VACV-G2 virus in 10 μL medium were applied to the dorsal ear skin and poked 25 times with a 27 ½ G needle. Thirty days post infection, skin resident memory recall experiments were performed.

Topical 4OHT Treatment. For topical 4OHT treatment, anesthetized K14 DIETER mice were treated on the ear with 2.5 μg of 4OHT (Sigma-Aldrich) in a solution of ethanol, water, and DMSO (48%/35%/17%) in a volume of 20 μL per ear. Mice were kept isolated for 4 h of incubation. Then, the treated skin was cleaned with a mixture of EtOH/water, and the mice were placed back in the original cages.

Topical IMQ Treatment. For topical treatment with IMQ, mice were anaesthetized and treated on the ear skin with 50 mg/ear of the IMQ solid nanoemulsion (IMI-Sol) containing 5% IMQ (provided by the group of P. Langguth, Department of Biopharmaceutics and Pharmaceutical Technology, Johannes Gutenberg-University Mainz) as described before (38).

Transcutaneous Immunization (TCI). For TCI, the previously published Dithranol–Imiquimod-based vaccination protocol was conducted (39). For topical treatments, mice were anaesthetized. For performing TCI, mice were treated on the ear skin with 50 mg/ear dithranol (0.625 μg/mg in Vaseline, manufactured by the Pharmacy of the UMC Mainz according to European Pharmacopoeia) and after 24 h with 50 mg/ear IMI-Sol (solid nano-emulsion containing 5% (w/w) IMQ). This was followed by treating the ear skin with 100 μg/ear GP_{33-41} peptide 100 μg/ear (Proteogenix, France), stirred into officinal cremor basalis. The development of an antigen-specific T cell immune response following TCI was monitored by tetramer-staining of CD8$^+$ T cells in the blood.

Diphteria Toxin Treatment. For depletion of DTR expressing Langerin$^+$ (CD207$^+$) cells from Langerin-DTR mice, mice were injected twice i.v. with 500 ng Diphteria toxin (Merck Millipore) in 100 μL Phosphate Buffered Saline (PBS) at the indicated timepoints.

Measurement of Ear Skin Thickness. A caliper (Mitutoyo) was used to measure the ear thickness. The mean of at least three measurements at different areas of the ear was calculated.

T Cell Isolation from Blood. Blood was collected from the submandibular vein using a lancet. After lysis of erythrocytes, the cell pellet was resuspended in PBS until further processing.

Generation of Single-Cell Suspensions from Whole-Ear Skin. For DC as well as T cell isolation from ear skin, the ear was split in its dorsal and ventral part and then cut into small pieces, followed by incubation in RPMI 1640 [without fetal bovine serum (FBS)] in the presence of 800 U/mL collagenase type IV (Worthington) and 50 U/mL DNase I (Sigma-Aldrich) for 90 min at 37 °C while shaking. Afterward, 0.5 M Sodium ethylenediaminetetraacetate (Na-EDTA) was added to a concentration of 10 mM and incubated further for 10 min. Cell suspensions were filtered through a 70-μm cell strainer.

Generation of Single-Cell Suspensions from Epidermis and Dermis of the Ear Skin. For the separation of epidermis from dermis, the ear skin was split in its dorsal and ventral part, with the dermal part facing down each sample was put for 45 min at 37 °C on the surface of digesting solution of 2.5 mg/mL Dispase II and 10 μg/mL DNase I in PBS. Then, the epidermal layer was peeled off the dermal layer. Each layer was cut in small pieces, followed by incubation in RPMI 1640 (without FBS) in the presence of 800 U/mL collagenase type IV (Worthington), and 50 U/mL DNase I (Sigma-Aldrich) for 90 min at 37 °C while shaking. Afterward, 0.5 M Na-EDTA was added to a concentration of 10 mM and incubated further for 10 min. Cell suspensions were filtered through a 70-μm cell strainer.

Purification of LC or Keratinocytes from the Epidermis. Dorsal and ventral ear halves were rinsed in 70% ethanol and air-dried for 5 min. After incubation with 2.5 mg/mL Dispase II and 50 U/mL DNase I for 45 min at 37 °C, the skin was separated into epidermis and dermis (58). For LC isolation, each epidermal sheet was then cultured, with the dermal part facing down, floating on 1.5 mL of complete RPMI-1640 containing 10% FBS in one well of a 24-well plate at 37 °C (59). After 48 h of culture, the emigrated cells were filtered via a 70-μm cell strainer, LC were enriched on density gradient centrifugation using OptiPrep, and then used for flow cytometric cell sorting (FACS). For keratinocyte isolation, epidermis was cut into small pieces and incubated in RPMI 1640 (without FBS) in the presence of 800 U/mL collagenase type IV (Worthington) and 50 U/mL DNase I (Sigma-Aldrich) for 90 min at 37 °C while shaking. Afterward, 0.5 M NaEDTA was added to a concentration of 10 mM and incubated further for 10 min. The obtained single-cell suspension was then filtered through a 70-μm cell strainer and used for FACS. LC were FACS sorted on living CD45$^+$, MHC-II$^+$, EpCAM$^+$ cells, and keratinocytes on living CD45$^-$ cells using an BD AriaII sorter. Cell fractions were then used in serial dilutions for coculture with naive T cells.

Nur77eGFPx327TCRxThy1.1 Coculture Assay. CD8$^+$ T cells were purified from the spleen of naive Nur77eGFPx327TCRxThy1.1 mice by magnetic cell separation using anti-CD8 microbeads with LS columns (Miltenyi). Then, 5×10^2 CD8$^+$ T cells were added to serial dilutions of APC in V-bottom 96-well plates. After 16 h of coculture, the TCR stimulation of Nur77eGFPxTCR327 T cells was assed by FACS based on their eGFP expression.

Antibodies and Flow Cytometric Analysis. Cells were stained for viability using fixable live dead stain efl780 (Thermo Fisher). Then, Fc-receptor blocks (2.4G2 and E9E) were applied. Fluorescently labeled antibodies used were CD4 (RM4-4), CD8 (53-6.7), CD11b (M1/70), CD11c (N418), CD19 (1D3), CD24 (M1/69), CD44 (IM7), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), CD69 (H1.2F3), CD62L (MEL-14), CD90.1 (OX-7), CD90.2 (145-2C11/17A2), CD103 (M290), CD172a or sirp1a (P84), CD326 or EpCAM (G8.8), H-2D^b (28–14–8), H-2 K^b (AF6-88.5), Ly-6C (HK1.4), Ly-6G (1A8), I-A/I-E or MHC-II (M5/114.152), NK1.1 (PK136), XCR1 (ZET). Flow cytometry was performed on a BD Canto equipped with three lasers (405 nm, 488 nm, and 640 nm) and BD Symphony with five lasers (355 nm, 405 nm, 488 nm, 561 nm, and 640 nm). Cell sorting was done on an BD AriaII with five lasers (375 nm, 405 nm, 488 nm, 532 nm, and 633 nm) using a 100-μm nozzle and a reduced flow pressure of 15 psi. Data were analyzed with FlowJo 10.8.1 (BD).

RNA Isolation from Murine Ear Skin Punches. Skin punches of 3 × 3 mm were harvested in Lysing D Matrix tubes (MP Biomedicals) and stored in TRI Liquid for RNA Extraction (Bio&Sell) at −80 °C until homogenization using a FastPrep-24® homogenizer. RNA was extracted from the homogenate according to the manufacturer's

instructions. Then, 300 ng of total RNA was reverse transcribed using RevertAid Reverse Transcriptase (Thermo Scientific) as per the manufacturer's instructions. RT-qPCR was performed on a StepOne System (Applied Biosystems) real-time PCR machine with EVAGreen® (Axon) reagent and the following primers: *Ifitm3* forward 5′ CTCTTCATGAACTTCTGCTGCC 3′, reverse 5′ CTCCAGTCACATCACCCACC 3′; *Ip-10* forward 5′ TTTCTGCCTCATCCTGCTG3′, reverse 5′ CTCATCATTCTTTTTCATCGTG 3′; *Hprt1* forward 5′ AACTTTGCTTTCCCTGGTTAAG 3′, reverse 5′ ATCCAACAAAGTCTGGCCTG 3′. Results were normalized to the housekeeping gene *Hprt1*. Fold differences in the transcription of IFNγ-induced genes between biological groups were determined using the comparative CT ($_{\Delta\Delta}$CT) method.

Generation and Immunostaining of Epidermal Sheets. The ears of mice were depilated using a hair removal cream. After washing in PBS, the ears were split into dorsal and the ventral part and fixed with the epidermal side on adhesive tape (Tesa Tape "crystal clear") (60). The skin samples were floated with the dermal side facing down on 20 mM EDTA in PBS for 90 min at 37 °C, then the dermis was carefully peeled off from the epidermis, which remained attached to the adhesive tape. The epidermal sheets were fixed for 15 min at room temperature using 4% Histofix, washed twice, and permeabilized using 0.1% Triton, followed by staining with directly conjugated antibodies [FITC-anti-MHC-II (M5/114.15.2), APC-anti-EpCAM (G8.8), and BV711-anti-Thy1.1. (OX-7)]. After washing, the sheets were mounted on slides with mounting media containing DAPI (Sigma-Aldrich).

Microscopy. The fixed and stained epidermal sheets were imaged on a Leica SP8 confocal microscope with a 20× 0.75 NA air objective sequentially with exposure from a 405-nm laser for transmission images and for DAPI/Hoechst (blue) excitation with emission and detection within a spectral window of 422 nm to 511 nm, with 638 nm laser exposure for APC (green) excitation with emission and detection within a spectral window of 647 nm to 700 nm and with 405 nm laser exposure for BV711 (red) excitation with emission and detection within a spectral window of 709 nm to 791 nm. The images were always acquired at a factor of at least 2.3 times less than the calculated confocal resolution, at a scan rate of 400 lines/min and with 2× averaging for 581 µm × 581 µm images with a pixel size of 0.11 µm (or 5,296 × 5,296 pixels). All images used in comparison were prepared and acquired under the same conditions. Control images acquired of just single-stained cells or of unstained cells revealed that under these detection and imaging schemes and under the conditions for imaging above that cross-talk fluorescence, background fluorescence and background autofluorescence were either nonexistent or so low that it would be statistically insignificant for any image of measurement. The images shown in the figures were smoothed with the standard Leica smoothing algorithm. In some cases, some signals had up to a 20% cutoff and/or a 20% threshold applied in order to remove background fluorescence and/or cellular autofluorescence and to create homogeneous cell borders. For acquisition, LasX Software was used and images were processed using ImageJ. For quantification of TCR327 T_{RM} in the epidermis, CD90.1$^+$ cells were counted manually per imaged area of five individual epidermal sheets. Of those identified CD90.1$^+$ cells, the frequency of cells in contact with the cell body or the dendrites of MHC-II$^+$ EpCAM$^+$ LC was determined.

Statistical Analysis. Statistical analysis was done with Prism 9 (GraphPad). Depending on the experiment, either one-way ANOVA (with Dunnet's post hoc test), two-way ANOVA (with Tukey's post hoc test), parametrical, or nonparametrical students *t* test (as indicated in the figure legends) was used to calculate the statistical significance of data.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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