

# External antigen uptake by Langerhans cells with reorganization of epidermal tight junction barriers

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**Outermost barriers are critical for terrestrial animals to avoid desiccation and to protect their bodies from foreign insults. Mammalian skin consists of two sets of barriers: stratum corneum (SC) and tight junctions (TJs). How acquisition of external antigens (Ags) by epidermal Langerhans cells (LCs) occur despite these barriers has remained unknown. We show that activation-induced LCs elongate their dendrites to penetrate keratinocyte (KC) TJs and survey the extra-TJ environment located outside of the TJ barrier, just beneath the SC. Penetrated dendrites uptake Ags from the tip where Ags colocalize with langerin/Birbeck granules. TJs at KC-KC contacts allow penetration of LC dendrites by dynamically forming new claudin-dependent bicellular- and tricellulin-dependent tricellular TJs at LC-KC contacts, thereby maintaining TJ integrity during Ag uptake. Thus, covertly under keratinized SC barriers, LCs and KCs demonstrate remarkable cooperation that enables LCs to gain access to external Ags that have violated the SC barrier while concomitantly retaining TJ barriers to protect intra-TJ environment.**

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Abbreviations used: 3D, three-dimensional; Ag, antigen; bTJ, bicellular TJ; KC, keratinocyte; LC, Langerhans cell; pAb, polyclonal antibody; SC, stratum corneum; SG, stratum granulosum; TJ, tight junction; tTJ, tricellular TJ; ZO-1, zonula occludens 1.

Although mucus covers the epidermis in fish and amphibian tadpoles, terminally differentiated cornified cellular sheets called the stratum corneum (SC) constitute the outmost epidermal layer in amphibian adults, reptiles, birds, and mammals and serve as a physical barrier to protect the living layer underneath (Madison, 2003). Beneath the mucus or SC, apical intercellular spaces of the living stratified epidermal cells are sealed with tight junctions (TJs) that limit paracellular leakage of water and electrolytes to maintain fluid homeostasis (Furuse et al., 2002; Tsukita and Furuse, 2002). The existence of TJ barriers in epidermis has been reported in fish, amphibians, reptiles, and, recently, in mammals (Farquhar and Palade, 1965; Mittal and Whitear, 1979; Landmann et al., 1981; Brandner et al., 2002). The knockout study of claudin-1, a TJ-specific integral membrane protein, demonstrated that TJs function as paracellular diffusion barriers in mammalian epidermis (Furuse et al., 2002). The lack of a wide-area visualization method for TJ honeycomb structures in skin, however, has hampered further detailed analysis.

Skin is a major entry site for microbial pathogens and allergens and is heavily guarded by DCs, leukocyte subsets which regulate immunity. Langerhans cells (LCs), which represent the most studied skin DCs and have been reported to elicit immune responses against foreign antigens (Ags) *in vivo* (Merad et al., 2008; Nagao et al., 2009), exist in the epidermis and, thus, are in the best position to encounter foreign Ags. LCs elongate their dendrites between keratinocytes (KCs) to comprise a dense network that covers the entire body surface. Routes of entrance for Ags, which are taken up by skin DCs, have not received attention in relation to skin barriers. Despite the discovery of mammalian epidermal TJs that had long been overlooked, this barrier is still totally neglected, and it is taken for granted that skin DCs have access to pathogens or allergens that have somehow

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entered the skin at the time of Ag uptake (McGrath and Uitto, 2008; Oyoshi et al., 2009). In this study, we demonstrate that activated LCs gain access to extra-TJ Ags by sending their dendrites out through epidermal TJs. TJ at KC–KC contacts allowed penetration of LC dendrite but maintained TJ barrier integrity by reorganizing new TJ at LC–KC contacts. Our study in stratified epithelium reveals a remarkably orchestrated system for Ag uptake and barrier maintenance that occurs at the forefront of external–internal interface in skin.

## RESULTS AND DISCUSSION

### Three-dimensional (3D) visualization of epidermal TJs and LCs

To study the functional interaction between the physical epidermal barrier and immunological surveillance system, we first enabled 3D visualization of TJs in epidermal sheets (Fig. 1 A and Video 1). 3D reconstruction images confirmed that the stratum granulosum (SG) is composed of three flattened KC cellular sheets (SG1, SG2, and SG3 cells, from top to bottom; Furuse et al., 2002; Tsuruta et al., 2002), and zonula occludens 1 (ZO-1) staining demonstrated that TJs seal the paracellular pathway between SG2 cells (Fig. 1 B and Video 1). Claudin-1 staining concentrated with ZO-1 in TJs but was diffuse on basolateral surfaces of SG2 cells and total cell surfaces of SG3 cells (Fig. 1 B and Video 1), which is concordant with previous results in vertical sections (Furuse et al., 2002). Note that SG1 cells are claudin-1 negative and exist outside of TJ barriers (Fig. 1 B; Video 1; and see Fig. 5 D).

We next evaluated the positioning of LC dendrites in relation to TJs. LCs could be visualized in conjunction with TJs in epidermal sheets with antibodies against ZO-1, claudin-1, and class II MHC (MHC II). MHC II was observed to accumulate in the perinuclear area of LCs (Fig. 1 C), suggesting that these LCs were in a resting state (Aiba and Katz, 1990). Claudin-1 staining exhibited a faint but well contrasted staining of TJs (Fig. 1 C, slice 7–23) and exhibited bright staining on the surface of LC cell bodies in the deep epidermis (Fig. 1 C, slice 43–51), which is consistent with previous FACS analysis reporting claudin-1 expression on LCs (Zimmerli and Hauser, 2007). 3D reconstruction images demonstrated an upward projection of LC dendrites toward the skin surface, which stopped short of the SG2 layer with obvious spaces between dendrite tips and TJs (Fig. 1 D and Video 2).

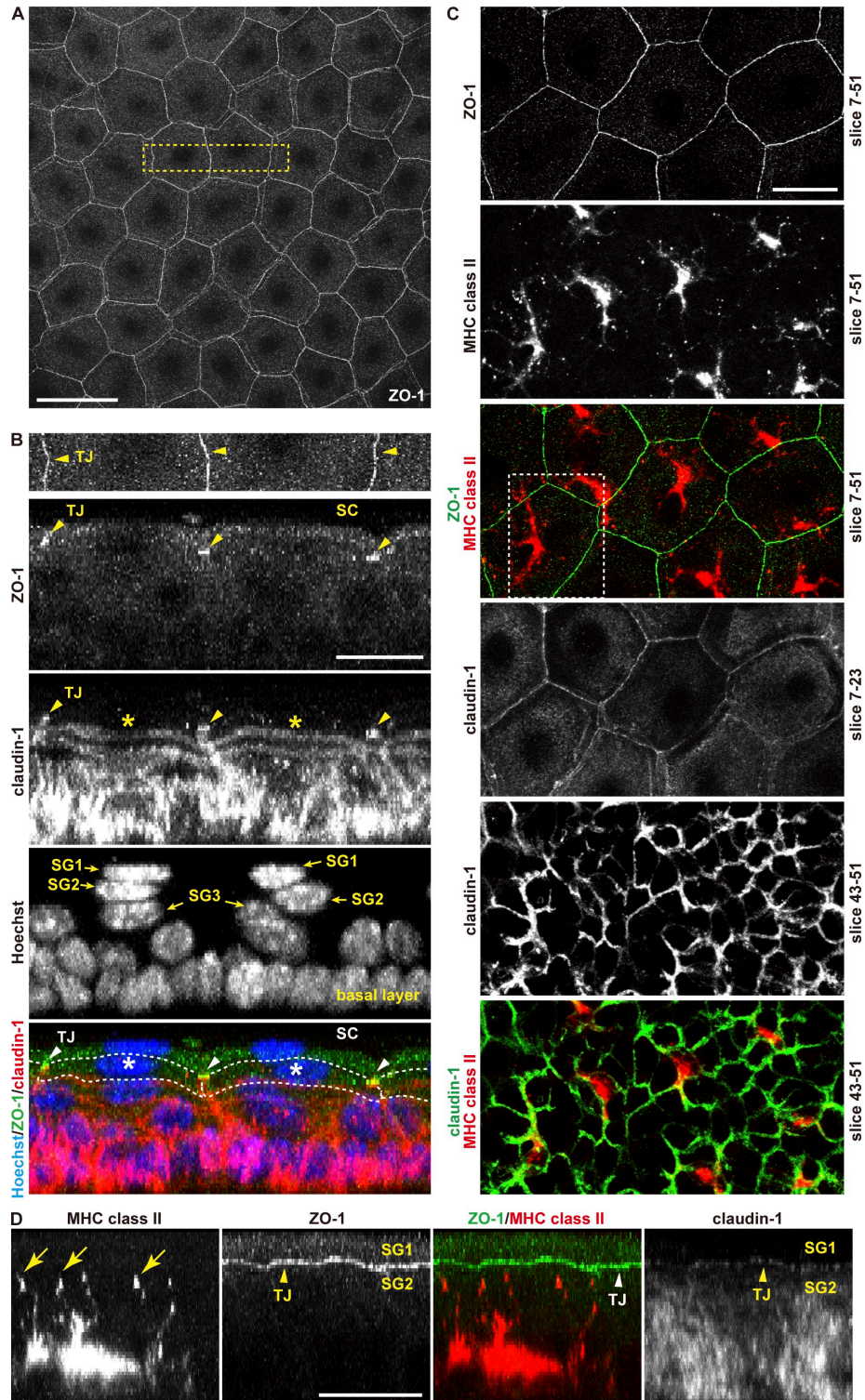
### Activated LCs elongate their dendrites through TJ

We then investigated the relationship between TJs and dendrites of activated LCs. Upon sensing inflammatory signals, LCs exhibit a series of coordinated changes, including increases in size, dSEARCH motion of dendritic processes, up-regulation of surface MHC II, and subsequent migration to draining lymph nodes (Thomas et al., 1980; Larsen et al., 1990; Nishibu et al., 2006). As LC activation is readily inducible *in vivo* by tape stripping (Streilein et al., 1982), we investigated the effect of tape stripping on TJs and LCs. SG1 cells and a significant portion of the SC remained intact after the tape-strip procedure as determined by confocal and electron microscopy (unpublished data). Tape stripping induced

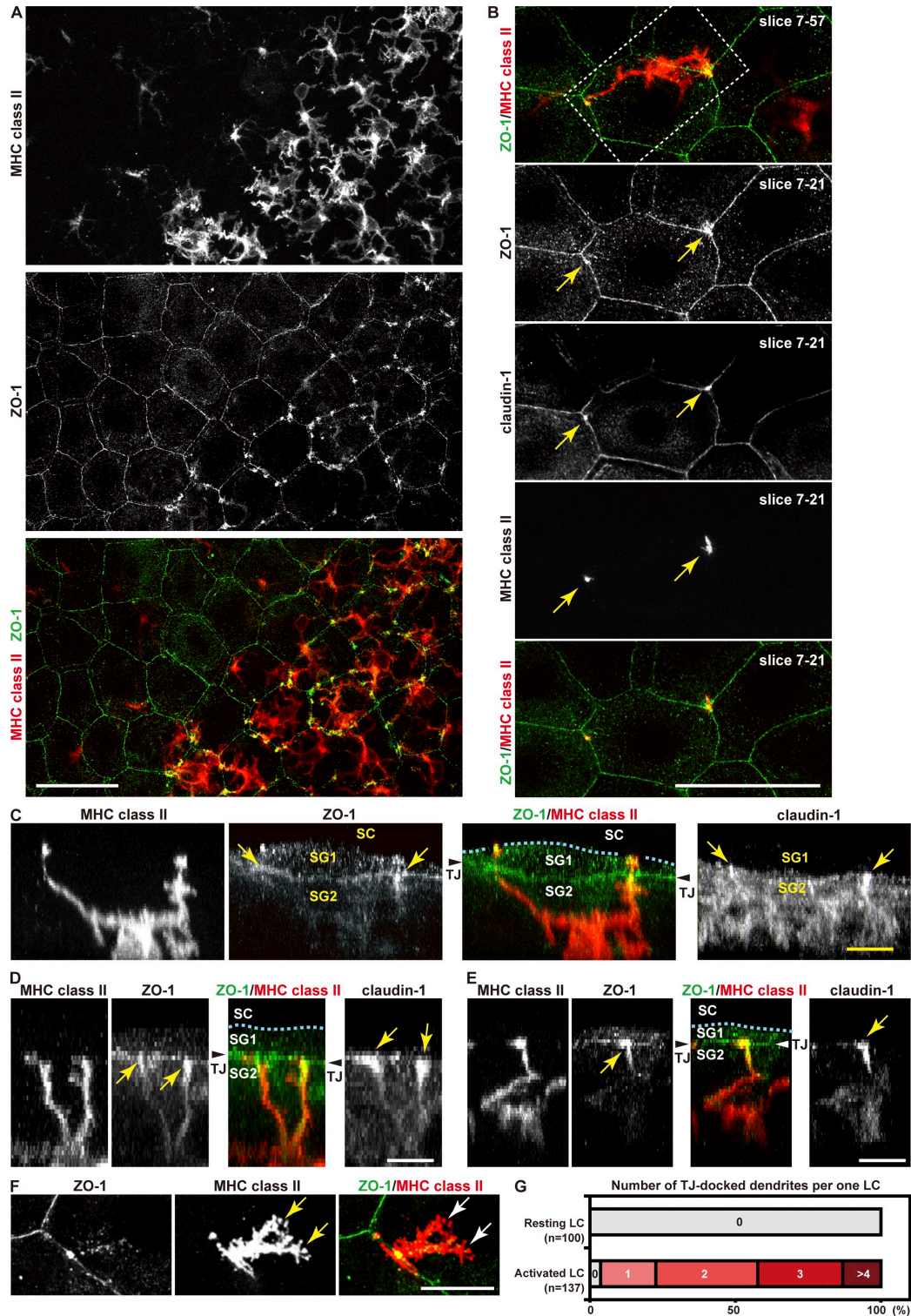
clusters of LCs to express high levels of surface MHC II within 12 h (Fig. S1 A and Fig. 2 A). We noticed that many ZO-1<sup>high</sup> spots were induced on TJs but were limited to those that overlaid MHC II<sup>high</sup> LCs (Fig. 2 A). High-magnification imaging of these ZO-1<sup>high</sup> spots revealed that MHC II<sup>high</sup> LC dendrites docked with TJs, indicating that the dendrite tips were exposed to extra-TJ environments of the SG1 layer (Fig. 2, B–E). Strikingly, some LC dendrites penetrated through TJs to reach the bottom of the SC (Fig. 2 C and Video 2). TJ docking or penetrating dendrites showed particularly high expression of ZO-1 and claudin-1 along their dendrites within the SG2 layer (Fig. 2, C–E), strongly suggesting that TJs are formed between LC dendrites and surrounding SG2 cells, presumably to retain TJ barrier integrity. After 12 h of tape stripping, the vast majority of activated LCs displayed one to four dendrites that had either docked or penetrated TJs (Fig. 2 G), both in ear and trunk skin (Fig. S2), indicating that intraepidermal activation of LCs almost always involves trans-TJ penetration of LC dendrites. Interestingly, LC dendrites have been reported to become highly mobile after tape stripping (Kissenpennig et al., 2005; Nishibu et al., 2006), indicating that activated LCs search paracellular spaces actively to dock their dendrites with TJs to reach the extra-TJ environment. Dendrite tips could sometimes be observed spreading horizontally between SG1 and SG2 cells after penetrating TJs, in which lamellipodia-like membrane protrusions were observed (Fig. 2 F), further suggesting that LC dendrites continue to search paracellular spaces after TJ penetration.

Next, we evaluated whether TJ docking and penetration of LCs occurred in response to cytokine signals. TNF and IL-1 $\beta$  have been reported to be KC-derived proinflammatory cytokines rapidly induced by tape stripping (Wood et al., 1992) and leading to LC activation and dSEARCH motion (Enk et al., 1993; Nishibu et al., 2007). As expected, subcutaneous injection of TNF or IL-1 $\beta$ , but not PBS, induced numerous clusters of MHC II<sup>high</sup> LCs within 12 h (Fig. S1 B). Again, these activated LCs uniformly exhibited TJ docking or penetration, and the number of TJ-docked dendrites was similar to that induced by tape stripping (Fig. S1 C). These observations demonstrated that TJ docking and penetration is a common feature for LCs that have undergone intraepidermal activation at least in an acute phase. Interestingly, genomic knockout of CX3CR1, which has been reported to mediate DC access to the intestinal lumen beyond TJs in lamina propria (Niess et al., 2005), failed to suppress tape stripping-induced TJ docking or penetration by LCs (unpublished data), suggesting the existence of unknown signals that remain to be identified.

Some LC dendrites were reported to end just beneath the SC, forming button-like structures in humans (Langerhans, 1868; Ferreira-Marques, 1951). Our study also showed that the dendrite tips at the TJ-docking site sometimes displayed button-like structures (Fig. 2 E). Perhaps the previous observations made by these LC pioneers were dendrites of activated LCs that had docked with TJs.



**Figure 1. 3D visualization of epidermal TJs and LCs.** (A) TJ network visualized by anti-ZO-1 antibody in epidermal sheet demonstrated en face by confocal microscopy. Bar, 50  $\mu$ m. (B) A 90° rotation image of the boxed area in A. The top shows an en face image of ZO-1 staining followed by rotation images stained for the indicated TJ molecules. Among the three layers of granular layer, SG1 to SG3, TJs (arrowheads) were found exclusively in the SG2 layer (asterisk and dashed lines), and rotation images display TJs as bright dots with intense ZO-1 and claudin-1 signals (see Video 1). (C) TJs and LCs in unperturbed skin. Z-slice numbers are indicated in each panel. ZO-1 and claudin-1 colocalized at KC-KC TJs, and LCs (MHC II<sup>+</sup>) coexpressed claudin-1 at high levels. (D) A 90° rotation image of the boxed area in C (see Video 2). Tips of LC dendrites (arrows) localized within TJ barriers (arrowheads). Bars, 20  $\mu$ m. Data presented is representative of five mice.



**Figure 2. TJ docking and penetration by activated LCs.** (A) Activated LCs exhibit brighter surface MHC II compared with resting LCs. ZO-1<sup>high</sup> spots identify where LC dendrites dock with TJs. (B) TJ penetration by activated LC dendrites (see [Video 2](#)) and accumulation of ZO-1 and claudin-1 at penetration points (arrows). Z-slice numbers are indicated in each panel. (C–F) Various forms of TJ docking and penetration. A 90° rotation of the boxed area in B is shown in C. Dashed lines indicate the SC–SG1 interface. Dendrites penetrated (C, arrows) or docked with (D, arrows) TJs, formed button-like structures (E, arrows), or formed lamellipodia-like protrusions (F, arrows) elongating horizontally between SG1 and SG2 cells after penetrating TJs. Bars: (A and B) 50 μm; (C–F) 10 μm. (G) Numbers of TJ-docked dendrites per cell on resting or activated LC after 12 h of tape stripping. Data presented in A–F is representative of five mice, and data in G of three independent experiments.

### TJ reorganization prevents TJ leakage at LC penetration points

Penetration of TJs at KC–KC contacts by LC dendrites presumably requires focal reorganization of TJ barriers that seal SG2 cells as well as concomitant formation of TJs at LC–KC contacts to minimize TJ leakage. TJ barriers in simple epithelia consist of bicellular TJs (bTJs) that seal intercellular space between two adjacent epithelial cells and tricellular TJs (tTJs) that form three pairs of vertically extended TJs at tricellular contacts (Fig. 3 A; Staehelin, 1973; Ikenouchi et al., 2005). Tricellulin is a transmembrane protein exclusively localized to tTJs and is indispensable for proper TJ barrier function (Ikenouchi et al., 2005). We determined that tricellulin also concentrates at tricellular points of TJs in stratified epithelia in vivo, further highlighting the importance of tTJs as a component of TJ barriers (Fig. 3, B and C). We hypothesized that if TJ integrity is maintained at TJ docking or penetrating sites, new tricellular contacts that form between an LC dendrite and two neighboring SG2 cells should be sealed by tTJs. Three patterns for docking could be modeled (Fig. 3 D, 1–3). Indeed, careful examination of TJ docking or penetration revealed all three patterns. In pattern 1 (Fig. 3 D, 1), in which dendrite penetrations were seen through KC–KC bTJs, two new sets of vertically extended LC–KC tTJs were revealed by tricellulin staining (Fig. 3 E and Video 3). In pattern 2, in which LC dendrites penetrate a preexisting KC–KC tricellular contact (Fig. 3 D, 2), three new LC–KC tTJs are positioned at nearly 120° intervals also on the newly formed LC–KC bTJ ring and extend vertically below (Fig. 3 F and Video 3). In pattern 3, a variation of pattern 2 (Fig. 3 D, 3), one vertical extension of tTJs managed to bring together four contact points (Fig. 3 G). The frequency of penetration through bicellular or tricellular KC–KC contacts was similar (Fig. 3 H). These observations strongly suggest that LCs and KCs actively interact with each other to strictly regulate the integrity of epidermal TJs during TJ penetration.

### TJ-penetrated dendrites engage in endocytosis

We next sought to address the biological significance of TJ penetration. If LCs uptake foreign material such as microbial pathogens before actual invasion without disabling the TJ barrier, it would be advantageous to the host. To address this, we investigated whether LCs could engage in endocytic activity via dendrite tips that have penetrated TJs. Langerin-positive Birbeck granule is an endocytic receptor that is, so far, unique to LCs (Valladeau et al., 2000). Langerin is a C-type lectin that binds mannose and related sugars in a calcium-dependent manner and has also been reported to mediate internalization of OVA and HIV (Valladeau et al., 2003; Takahara et al., 2004; Merad et al., 2008). We found that langerin accumulated in dendrite tips of activated LCs but not in resting LCs (Fig. 4 A), indicating that Birbeck granule formation is prominent in activated LC dendrites.

To analyze endocytic activity, we topically applied a protein biotinylation reagent, sulfo-NHS-LC-biotin, on mouse ear skin. This reagent is membrane impermeable and epidermal TJ unpenetrable (Furuse et al., 2002). Remarkably, 16 h

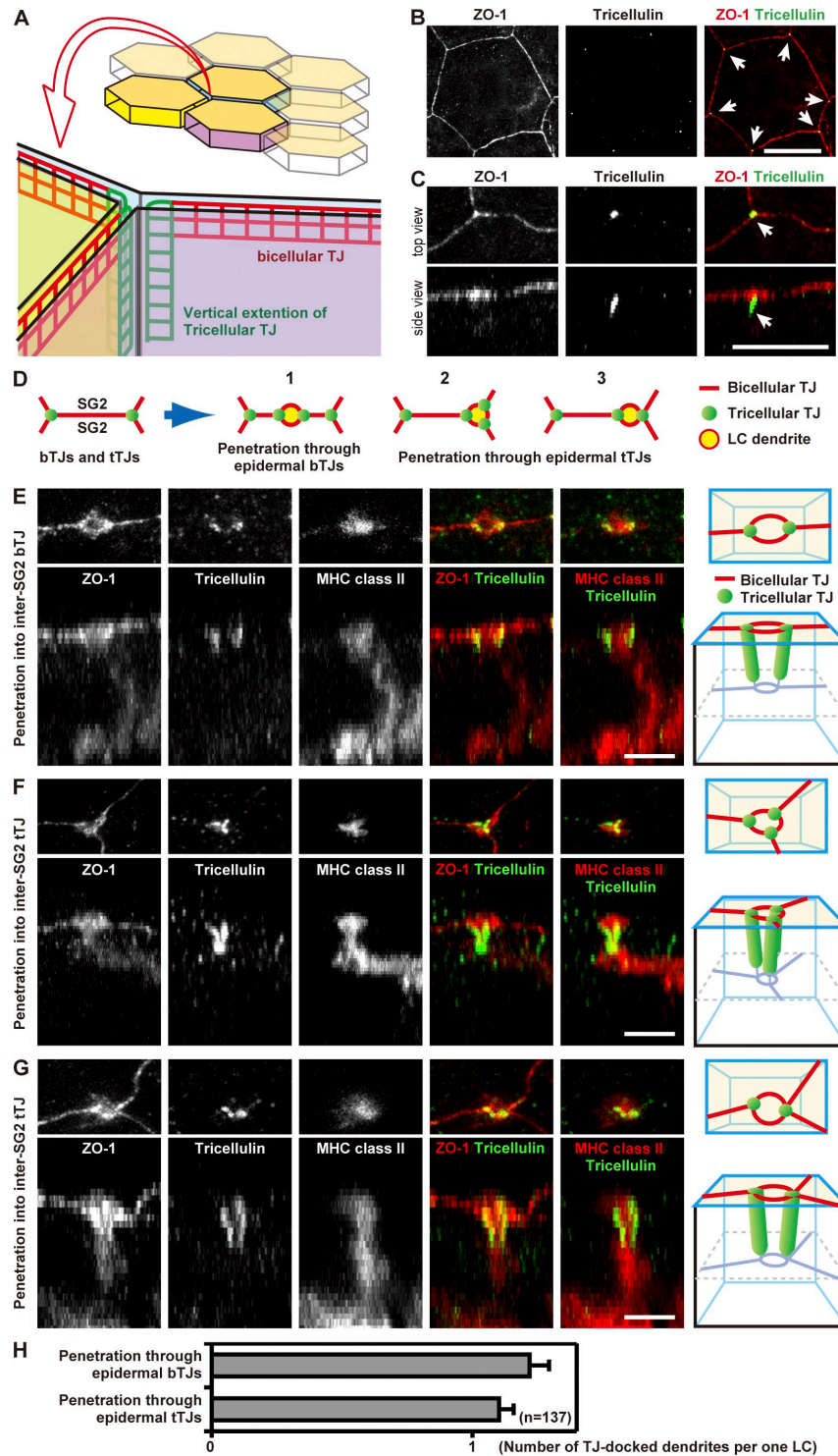
after application, biotin signals accumulated within dendrites of activated LCs that were limited to those that had penetrated TJs (Fig. 4 B and Video 4). Biotin signals were observed as a streak starting from the tip through the center of the dendrites (Fig. 4 C and Video 4), indicating that active endocytosis takes place at the tip, and there exists a continuous membrane flow to the cell body. A significant part of biotin signals within dendrites colocalized with langerin (Fig. 4 D), indicating that Birbeck granules take part in this endocytic machinery.

### LCs uptake extra-TJ Ags

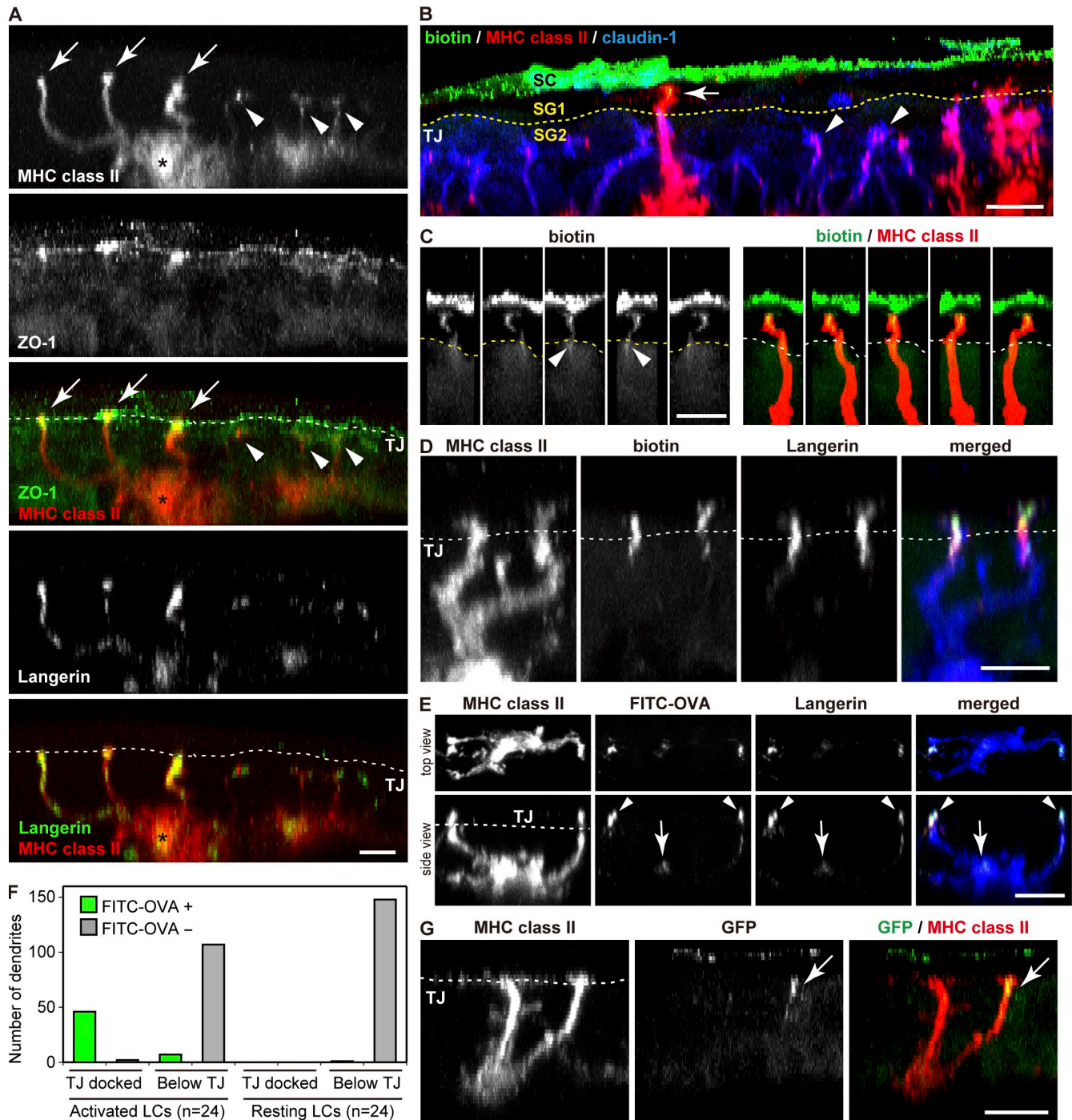
Previous in vitro studies demonstrated that langerin could mediate uptake of OVA (Takahara et al., 2004). To directly determine that the endocytic machinery described in the previous section is associated with Ag uptake, we examined whether LCs were capable of acquiring foreign Ags in vivo using FITC-OVA. Because an aqueous FITC-OVA solution failed to pass through the SC by simple topical application, we applied it using an occlusive dressing. TJ is a size-selective barrier. In good agreement with this, a 45-kD FITC-OVA signal was not observed in the living layer of epidermis (Fig. 4 E). Strikingly, FITC-OVA was observed to accumulate within the LC dendrites that penetrated TJs but not within dendrites that stayed below TJ barrier, even in activated LCs (Fig. 4, E and F). In TJ-penetrated LCs, langerin colocalized with FITC-OVA signal that displayed as streaks in dendrites and as perinuclear accumulations, strongly suggesting that FITC-OVA may be endocytosed via Birbeck granules at the tip and transported by intracellular trafficking (Fig. 4 E and Video 4). Application of GFP-expressing *Escherichia coli* resulted in strong GFP signal in TJ-penetrating dendrites, indicating that skin surface microbes or associated Ags could also be captured (Fig. 4 G).

Finally, we performed electron microscopy using lanthanum nitrate to confirm the existence of Birbeck granules at TJ-penetrating tips and to confirm the integrity of TJs where penetration occurs (Fig. 5, A–C). The lanthanum penetration assay is an established method to evaluate TJ permeability (Hashimoto, 1971; Shaklai and Tavassoli, 1982). Indeed, some LC dendrites were found in the extra-TJ environment above SG2 (Fig. 5 B), where Birbeck granule formation was confirmed on the cell membrane of these penetrating dendrites (Fig. 5 C), further enforcing our confocal microscopy data. Fig. 5 A shows that diffusion of lanthanum nitrate is limited by TJs that seal SG2 cells. At the TJ penetration point, contact between LC and SG2 cells was also capable of stopping lanthanum diffusion, which was confirmed in serial ultrathin sections (Fig. 5 B, representative electron micrograph), indicating the presence of functional TJs between LC and SG2 cells.

Collectively, epidermal LCs elongate their dendrites through TJ barriers upon activation and engage in endocytic activity across TJ barriers. Taken-up Ags colocalized with langerin, strongly suggesting the involvement of Birbeck granule-associated mechanisms during this endocytic machinery (Fig. 5 D).



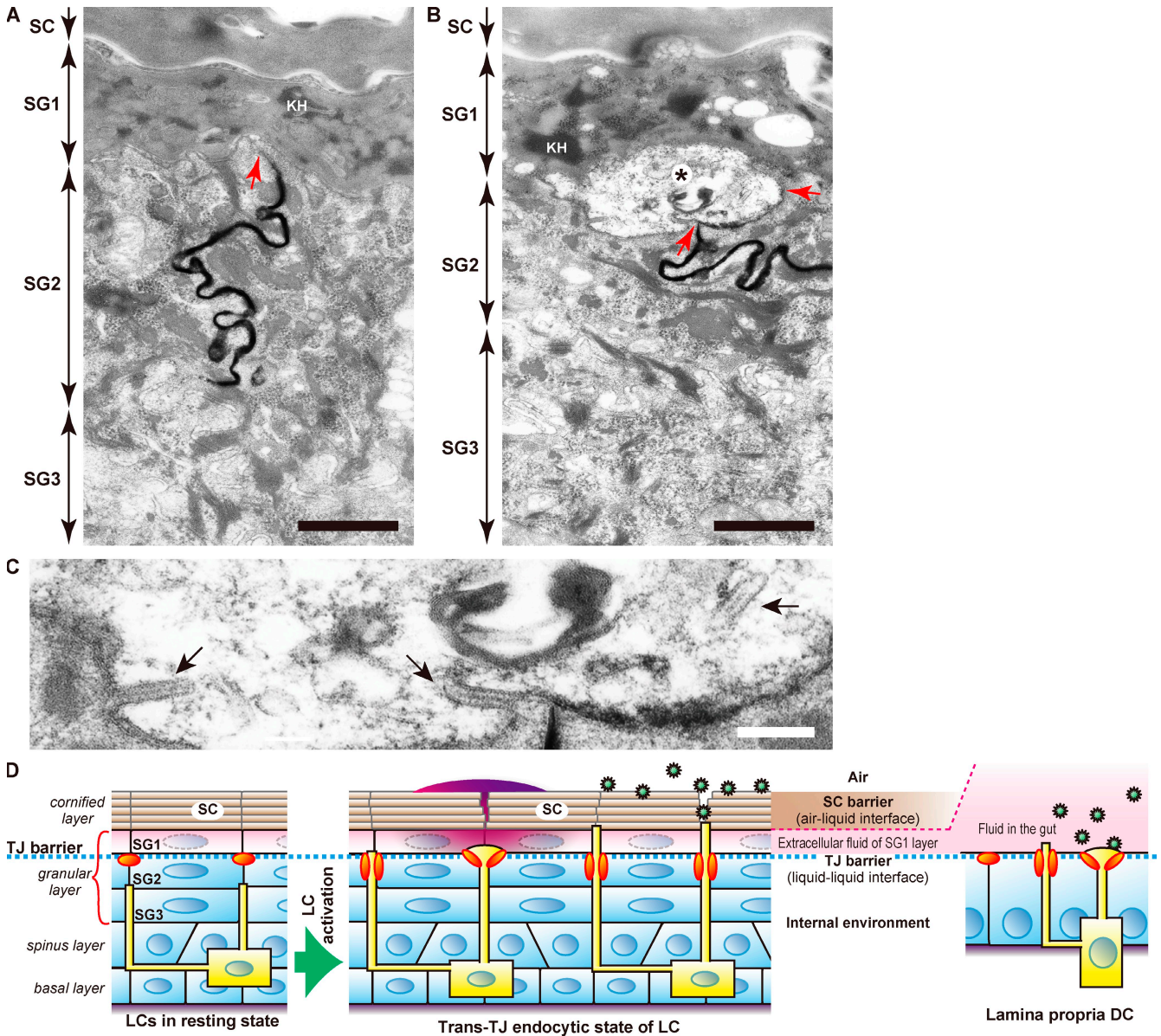
**Figure 3. tJ formation at the TJ penetration point of LC dendrites.** (A) Schematic structure of TJs at cell–cell contacts. TJ strands extend vertically below at tricellular contacts to form tTJs (Ikenouchi et al., 2005). (B and C) 3D reconstruction of epidermal sheet stained for ZO-1 and the tTJ protein tricellulin. Tricellulin appears as bright dots (B, arrows) when observed en face, but is found to extend vertically below in 90° rotated images (C, arrows). Bars, 20  $\mu$ m. (D) Schematic representation of three patterns by which LC dendrites penetrate bTJs or tTJs at the SG2 layer. (E–G) 3D reconstruction images of LC dendrites penetrating TJs as modeled in D (see Video 3). Top rows of each show en face images and bottoms present 90° rotation images accompanied by schematic drawings on the right of each row. Bars, 5  $\mu$ m. (H) Numbers of bTJ- and tTJ-docked dendrites per LC. The number of dendrites that docked with or penetrated TJs was counted on activated LCs after 12 h of tape stripping. Error bars represent SEM. Data presented in A–G is representative of three mice and data in H of three independent experiments.



**Figure 4. LCs engage in trans-TJ endocytosis via langerin.** See Video 4 for more details. (A) Visualization of langerin and MHC II on activated (asterisks) and resting (cell to the right) LCs in relation to TJs as shown by ZO-1 staining. Dashed lines indicate TJs. Langerin accumulated in TJ-docked dendrite tips of activated LCs (arrows). Dendrites of resting LCs (arrowheads) lack langerin accumulation. (B) Biotinylation of LC surface membrane reveals endocytic activity by a TJ-penetrated dendrite (arrow). Dashed lines represent TJs. Dendrites of resting LCs (arrowheads) lack biotin signals. (C) Rotated views of an endocytosing LC dendrite from B. The streak of biotin signal in the dendrite continues within the TJ barrier (arrowheads). (D) Colocalization of biotinylated molecules (green) with langerin (red) in TJ-penetrated dendrites as shown by MHC II (blue) staining. (E) FITC-OVA (green) application demonstrates trans-TJ uptake of Ags by LCs in vivo. FITC-OVA signals colocalized with langerin (red) in TJ-penetrating dendrites (MHC II, blue; arrowheads). Arrows point to accumulation of FITC and langerin signals in perinuclear area of an activated LC. (F) Numbers of FITC-OVA-positive and -negative dendrites. The number of dendrites that docked or did not dock with TJs was counted on resting and activated LCs. (G) GFP-expressing *Escherichia coli* application demonstrates GFP signal within LC dendrite (arrows). Bars, 10  $\mu$ m. Data presented in A–E and G is representative of three mice and data in F of three independent experiments.

TJ formation between distinct lineages of cells had been poorly described. This study demonstrated that reorganization of epidermal TJ barriers is a dynamic process involving intimate interactions between LCs and the SG2 layer of KCs. This interaction allowed LCs to penetrate their dendrites through TJs, enabling uptake of external Ags in the presence of intact TJ barriers. Tape stripping immediately induced a series of these events and subsequent emigration of activated LCs from

the epidermis after 48 h (Fig. S1 A). This indicated that the intraepidermal activation and sequential migration is a well orchestrated biological process for LCs to take up Ags that exist beyond intact TJ barriers without allowing their invasion and, presumably, to present them to the immune system. To our interest, the existence of MHC II-positive intraepidermal DCs has been reported not only in mammals but also in amphibians, reptiles, and birds (Akhter et al., 1993; Pérez-Torres and



**Figure 5. Penetrating dendrites and KCs retain TJ barrier function.** Electron microscopy images of SC, SG cells, and dendrite tip of an LC. Keratohyalin granules (KH) identify SG1 cells. (A) Lanthanum permeability assay. Intercellular diffusion of lanthanum nitrate (electron-dense streak) is blocked at KC-KC TJs (arrow). (B) TJ-penetrated LC dendrite (asterisk) was found between the SG1 and SG2 cell layers. The junction between the LC dendrite and SG2 cells also inhibited lanthanum diffusion (arrows), suggesting an intact TJ barrier. Bars, 500 nm. (C) Enlarged image of the LC dendrite tip (B, asterisk), in which two of three Birbeck granules (arrows) were observed to generate from the cell membrane. Bar, 100 nm. (D) Schematic model for trans-TJ uptake activity of LCs. Activation of LCs induces elongation of LC dendrites beyond TJ barriers. bTJs and tTJs that are newly formed between LC and SG2 cells preserve the integrity of TJ barriers during this phenomenon. LCs access Ags that have violated the SC barrier in the presence of intact TJ barrier function. Schematics for gut intraepithelial DCs called lamina propria DCs are shown for comparison. Data presented in A–C is representative of two mice.



Millán-Aldaco, 1994; Pérez-Torres et al., 1995; Castell-Rodríguez et al., 1999). Future study will be needed to clarify that Ag uptake across epidermal TJ barrier by intra-epidermal DCs is an evolutionarily conserved defense system of the skin.

Our findings emphasize the resemblance of the surface barrier system between the epidermis and the gut. In intestinal mucosa, lamina propria DCs have been reported to elongate their dendrites directly into gut lumen to sample bacteria (Rescigno et al., 2001), suggesting that dynamic reorganization of TJs, similar to our finding, occurs between gut DCs and TJs. In comparing these two barrier interfaces, although the surface of the gut epithelial cells that are sealed by TJs is covered by fluid and mucus, which constitutes a liquid–liquid interface system, the surface of epidermis is covered by SC to protect the body from desiccation and coarse microbes or allergens and constitutes an air–liquid interface system (Fig. 5 D). Our findings demonstrate that underneath the SC, but outside the epidermal TJ barriers, is a layer of viable cells called SG1 cells which are likely to be soaked in extracellular fluid. Therefore, similar to the gut, TJs in skin also serve as a liquid–liquid interface barrier. The SG1 layer has never received much focus with regard to barrier function in skin. It appears to be an important layer by not only providing an environment for LCs to take Ags across TJ barriers but also to serve as an intermediate layer that bridges air–liquid and liquid–liquid interfaces (Fig. 5 D).

Our future investigations on the trans-TJ endocytic activity of LCs together with the epidermal barrier system will receive greater focus on the mechanisms or signals that initiate TJ penetration, as well as immunological consequences of this process. This focus should provide new insights not only into pathological conditions such as contact hypersensitivity or chronic atopic dermatitis but should also be of particular interest in the context of antiinfection immunity and percutaneous vaccination.

## MATERIALS AND METHODS

**Animals.** Female C57B6/J 8–12-wk-old mice were used in all experiments. All animal protocols were approved by the animal ethics review board of Keio University and conformed to the National Institutes of Health guidelines.

**Immunofluorescence and confocal microscopy.** Epidermal sheets were prepared from the ventral side of the mouse ear skin or mouse trunk skin and immunostained as described previously with some modifications (Shelley and Juhlin, 1979; Kubo et al., 2008). Samples were mounted in a whole-mount fashion using Mowiol (EMD) and observed under a fluorescence microscope (BZ-9000; Keyence) equipped with a 20× objective or a laser-scanning confocal microscope (TCS sp5; Leica) equipped with a 63× objective using 0.4–0.5- $\mu$ m optical slices. 3D reconstruction images were built using sp5 software (Leica). Images and movies were processed using Photoshop CS4 (Adobe), Illustrator CS4 (Adobe), and QuickTime Pro (Apple).

**Antibodies.** The following antibodies were used in these experiments: anti-claudin-1 polyclonal antibody (pAb; Abcam and Invitrogen), anti-ZO-1 mAb (clone T4-192; Itoh et al., 1991; provided by M. Furuse, Kobe University, Kobe, Japan), anti-tricellulin pAb (Invitrogen), anti-MHC IA/IE mAb (eBioscience), biotinylated anti-MHC IA/IE mAb (eBioscience), Alexa Fluor 488-conjugated anti-GFP pAb (Invitrogen), and anti-mouse Langerin pAb (Imgenex). Species-specific secondary antibodies labeled with Alexa Fluor 488, 568, and 647 (Invitrogen) were used for detection. Biotin-

ylated proteins were detected by Alexa Fluor 568-conjugated streptavidin (Invitrogen), and cell nuclei were stained with Hoechst 33258 (Invitrogen).

**LC activation experiments.** Ventral sides of mouse ears were used in all experiments. C57BL/6J mice were anaesthetized by intraperitoneal injection of pentobarbital sodium (nembutal; Dainippon Sumitomo Pharma) before LC activation procedures. In tape-stripping experiments, whole-skin surfaces of ventral ears were subsequently tape stripped using scotch tape three times. In cytokine-induced LC activation studies, 50 ng TNF- $\alpha$  (PeproTech) or 50 ng IL-1 $\beta$  (PeproTech) in 30  $\mu$ l PBS containing 0.7 mM CaCl<sub>2</sub> and 0.1% bovine serum albumin (Sigma-Aldrich) or 30  $\mu$ l of buffer alone was injected subcutaneously into the ventral ear. In topical application experiments, 100  $\mu$ l of 10 mg/ml EZ-link sulfo-NHS-LC-biotin (Thermo Fisher Scientific) in PBS containing 1 mM CaCl<sub>2</sub> was applied to the surface of ventral ear skin. FITC-OVA was delivered by the occlusive dressing technique, in which a small piece of filter paper was moistened by 20  $\mu$ l PBS or 10  $\mu$ g FITC-OVA in 20  $\mu$ l PBS solution and applied to mouse ear skin with Finn chambers (SmartPractice). Finn chambers were covered with adhesive bandages to prevent the mice from scratching them. GFP-expressing *Escherichia coli* was produced by transforming BL21 strain of *Escherichia coli* with pGFP vector (Takara Bio Inc.). GFP-expressing *Escherichia coli* was applied by occlusive dressing technique on ventral ear skin that was tape stripped three times before application. Skin was harvested after the 24-h occlusive application of FITC-OVA or GFP-labeled *Escherichia coli*. It was confirmed that occlusive dressing of PBS alone induced LC activation in situ.

**Electron microscopy.** Mouse ears were tape stripped 12 h before harvest. Ventral and dorsal sides of the ear were split and the cartilage was removed and fixed with 2.5% glutaraldehyde containing 1% lanthanum nitrate and prepared as described previously (Hashimoto, 1971). Samples were examined with an electron microscope (H-7500; Hitachi High-Technologies) at the accelerating voltage of 80 kV.

**Online supplemental material.** Fig. S1 shows the time course of LC activation after tape stripping and LC activation induced by subcutaneous injection of TNF- $\alpha$  or IL-1 $\beta$ . Fig. S2 shows TJ docking by activated LCs in mouse trunk skin. Video 1 shows 3D visualization of epidermal structure and TJs in mouse ear skin. Video 2 shows that activated LCs, but not resting LCs, elongate their dendrites and penetrate the TJs. Video 3 shows penetration of LC dendrites through bicellular and tricellular junctions between KCs. Video 4 shows trans-TJ endocytic activity and trans-TJ Ag uptake of LCs. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20091527/DC1>.

We dedicate this work to the memory of Professor Shoichiro Tsukita.

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