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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Human type 2 innate lymphoid cells disrupt skin keratinocyte tight junction barrier by IL-13

To the Editor,

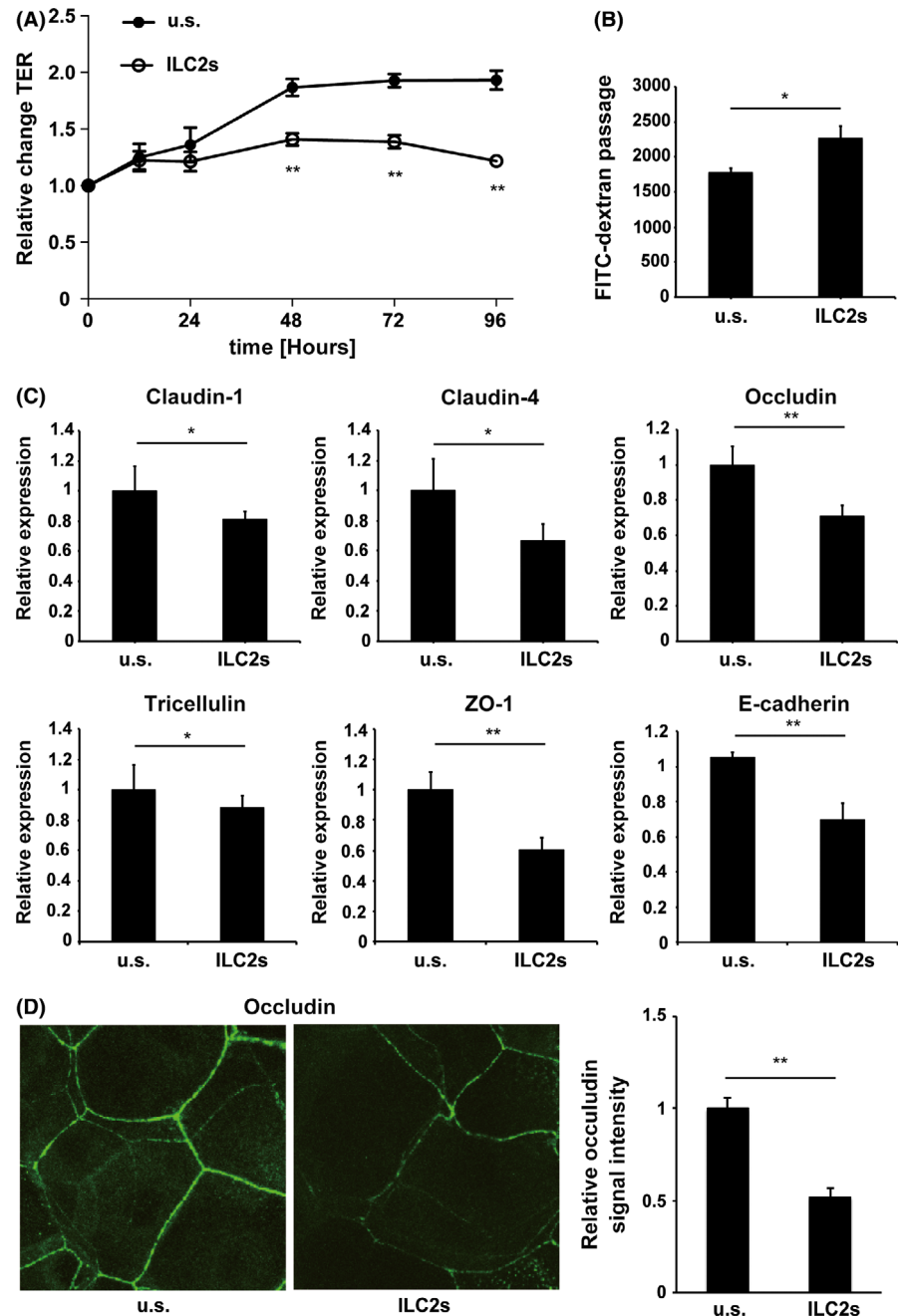
The largest organ of the human body is the epidermis, which consists mainly of keratinocytes (KCs) and serves as a physical barrier. Along with the stratum corneum, which is the outer surface of the epidermis, the tight junctions (TJs) located in the skin surface side of stratum granulosum are crucial for the integrity and function of the epidermal barrier.^{1,2} TJs form a network of molecules that ensures a nonpermeable intercellular adhesion and seal the paracellular space in the epithelium, thus protecting the body from the penetration of invading microorganisms, pollutants, environmental toxins, and allergens.³ Accordingly, skin KC TJ barrier dysfunctions permit the penetration of antigens, allergens, toxins, and pollutants through the surface of the epidermis to dermis and subdermal connective and fat tissues. Recent evidence suggests that TJ barrier dysfunction plays a key role not only in atopic dermatitis but also in asthma, allergic rhinitis, chronic rhinosinusitis, and colitis.⁴ Other data from human and animal studies have shown that impairment of the skin barrier is an important mechanism in allergen sensitization.⁴ Allergic sensitization triggers the first induction of innate immune responses by pathogen recognition receptors on epithelial cells and immune cells, such as innate lymphoid cells (ILCs), dendritic cells, macrophages, and mast cells, and leads to subsequent activation of type 2 immune responses.⁴

Innate lymphoid cells are preferentially enriched in barrier tissues such as the skin, lung, and intestine and play important roles in the maintenance of tissue homeostasis, regulation of immune

responses between epithelium and microorganisms, and sensitization to allergens.⁵ Among ILC subsets, type 2 innate lymphoid cells (ILC2s) reside in the barrier surfaces and contribute to lung inflammation and asthma development.⁶ They enable the immune system to respond quickly to environmental antigens on epithelial surfaces. Mechanisms of skin and mucosal barriers differ, and skin shows a stronger barrier both with filaggrin, loricrin, involucrin located in the stratum corneum, and TJs located in the stratum granulosum. We have recently demonstrated that ILC2s facilitate bronchial epithelial barrier disruption via downregulation of the TJ barrier through IL-13 in asthma.⁷ Although ILCs are relatively well studied with regard to their role in the development of skin diseases such as atopic dermatitis and psoriasis, several major questions remain to be addressed concerning whether the epidermal TJ barrier is influenced by ILC2s.

In the present study, we first investigated the role of ILC2s in the regulation of TJ function of human KCs. We cultured normal human epidermal keratinocytes (NHEKs) with ILC2s at air-liquid interface (ALI), and transepithelial resistance (TER) was measured as a readout for barrier integrity (See Online Supporting Information). Data analysis was performed using 2-tailed Mann-Whitney *U* test or analysis by variance (ANOVA), followed by Tukey's post hoc multiple comparison. A *P* value of <0.05 was considered significant. ILC2s were cocultured with NHEKs and TER was measured before and after 12, 24, 48, 72, and 96 hours. We found that after 48 hours of coculture with ILC2s a significant decrease in TER was observed (Figure 1A). To further investigate whether ILC2s

FIGURE 1 ILC2s disrupt normal human epidermal keratinocyte (NHEK) barrier. A, ILC2s decreased TER after coculture with human NHEKs. *us*, unstimulated. B, Increase in FITC-dextran paracellular permeability across NHEKs cocultured with ILC2s. *us*, unstimulated. C, mRNA expression of tight junction (TJ) genes cocultured with ILC2s. The results indicate significantly lower expression of TJ genes in ILC2s than in control cells ($n = 3$). *us*, unstimulated. A-C, Data were obtained from three independent experiments performed in triplicates. D, Immunofluorescence staining of occludin in NHEK-ILC2 cocultures. Decreased immunofluorescence detection of occludin in ALI-cultured NHEKs treated with ILC2s. Cells were fixed after 96 h with ILC2s. Representative pictures from three independent experiments are shown and fluorescence intensities from five different microscopy fields were compared with the ImageJ software. *us*, unstimulated. Values are means $\pm$ SEMs in Figure 1, A-D, * $P < 0.05$ and ** $P < 0.01$



influenced keratinocyte permeability for large molecules, we measured paracellular permeability by analyzing fluorescein isothiocyanate (FITC)-conjugated 4-kDa dextran's paracellular passage across keratinocyte layers. Coculture of NHEK with ILC2s for 96 hours significantly increased the passage of fluorescently labeled dextran across the epithelial layers (Figure 1B). Paracellular permeability was dose-dependently linked to the presence of ILC2s cocultured with KCs both in TER (Figure S1) and macromolecular permeability assays (Figure S2). These data suggest that ILC2s disrupt keratinocyte barrier integrity.

To further investigate skin keratinocyte TJ barrier by ILC2s, we analyzed the mRNA expression of TJ and related genes by quantitative PCR. The expression of claudin-1, claudin-4, occludin,

tricellulin, zonula occludens 1(ZO-1), and E-cadherin was down-regulated after coculture with ILC2s for 12 hours, indicating that the influence of ILC2s on KCs was exerted at the gene-expression level of TJ-related molecules (Figure 1C). TJ proteins must be correctly assembled into TJ structures to efficiently contribute to the barrier function of KCs. Here, we investigated the distribution of the TJ molecule occludin after coculture with ILC2s. The immunostaining pattern of occludin was markedly perturbed in the KC-ILC2 cocultures at 96 hours with lower intensities compared with control cells (Figure 1D). These changes in immunofluorescence staining suggest that the reduced protein expression of occludin may be linked to impaired TJ barrier integrity. Thus, together with decreased TER, the observed damage to TJ molecules

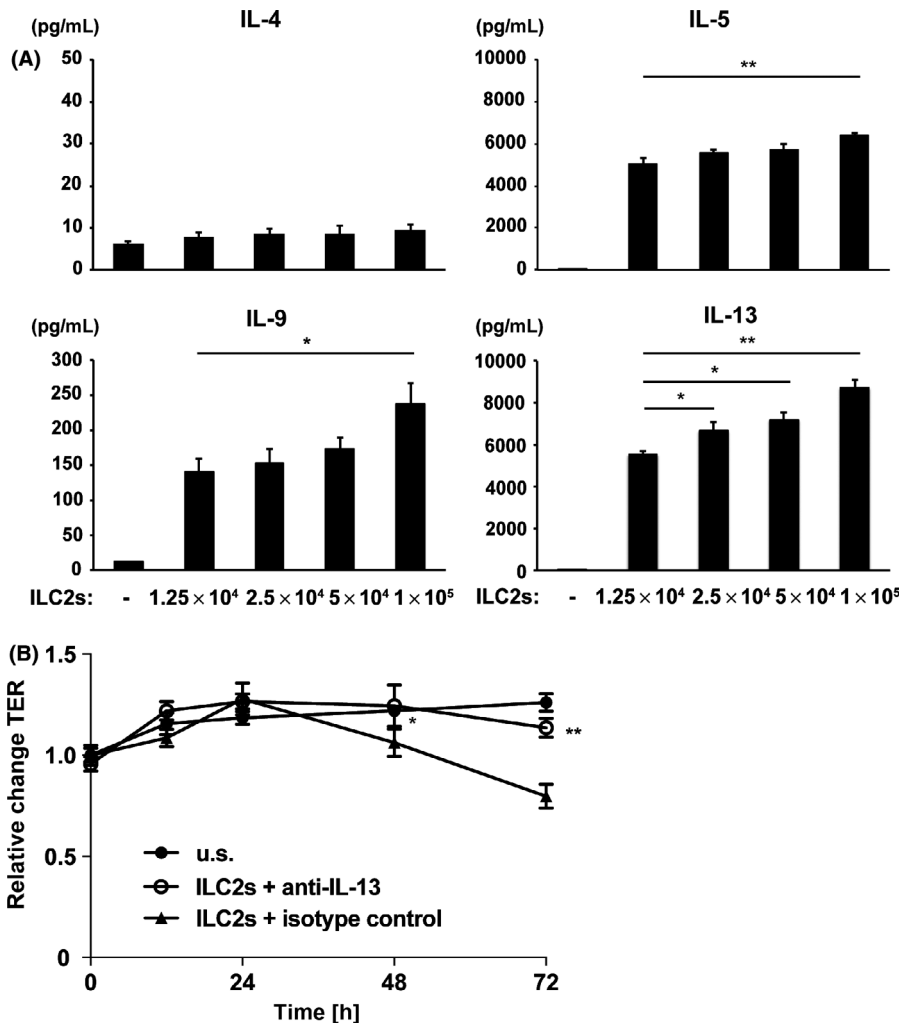


FIGURE 2 Th2 cytokine production by NHEK-ILC2 cocultures. A, Production of IL-5, IL-9 and IL-13 by human ILC2s was increased in an ILC2-number dependent manner after 72 h of NHEK-ILC2 coculture. Cytokine secretion was quantified in NHEK coculture supernatants by means luminometric multiplex cytokine quantification. Representative data are shown (n = 4). B, Effects of neutralization of IL-13 on TER. Anti-human IL-13 antibody (Source, polyclonal goat IgG; R&D Systems), at a concentration of 10 μ g/mL, restored the barrier function, which was damaged by ILC2. As isotype control normal goat polyclonal goat IgG manufactured from the same company was used at the same concentration. Effects of neutralization of IL-13 on TER were comparable to the unstimulated control. A and B, Data were obtained from two independent experiments in quadruplicates (A) and triplicates (B). *us*, unstimulated. Values are means $\pm$ SEMs in Figure 2, A and B, **P* < 0.05 and ***P* < 0.01

is linked to decreased barrier integrity and increased paracellular permeability.

Because ILC2s are an important source of type 2 cytokines,⁵ we further analyzed the cytokine production from ILC2s in dose-response experiments. NHEKs were cultured alone or with ILC2s in different conditions for 72 hours. As shown in Figure 2A, ILC2s produce high amounts of IL-5, IL-9, and IL-13 in a dose-dependent manner. Since IL-5 and IL-9 did not affect barrier function in KCs (Figure S3), we neutralized IL-13 to demonstrate its major role for barrier disruption. Interestingly, the neutralization of IL-13 in ILC2s cocultured with KCs restored the barrier function that was otherwise disrupted by ILC2s (Figure 2B). In our ILC2-KC coculture, the number of 1×10^5 -ILC2s produced about 10 ng/mL IL-13. Based on the manufacturer's data, the concentration of anti-IL-13 antibody at 10 μ g/mL is an adequate concentration to completely neutralize IL-13.⁷ We investigated whether IL-13 affects barrier function of normal human epidermal keratinocytes (NHEKs). As shown in Figure S4, NHEKs exposed to IL-13 showed significantly lower TER compared with unstimulated NHEKs, suggesting that IL-13 recapitulates the decreased barrier integrity by ILC2-derived IL-13. Taken together, these results indicate a potential role of ILC2s and IL-13 in skin barrier disruption by targeting TJ.

New insights into atopic dermatitis revealed an important role for impaired barrier integrity in the epidermis resulting in a leaky epithelial barrier as well as chronic immune activation.^{4,8} Skin barrier perturbations and immunological abnormalities interact with each other in the pathogenesis of AD and have raised the question whether ILC2-related cytokines affect skin barrier TJs. Accordingly, we demonstrated that NHEK cocultured with ILC2s remarkably attenuated the TJ barrier through IL-13. We speculate that activation of ILC2s under the culture conditions can effectively produce IL-13 in vitro. We measured the amount of IL-13 from the ILC2 cultures of four different donors, at 72 hours (9968 ± 2903 pg/mL, n = 4). The data indicate that the amount of IL-13 production is different between blood donors, suggesting that different amounts of IL-13 in KC-ILC2 coculture condition may affect the barrier function of KCs, resulting in differences in barrier disruption levels of KCs in response to ILC2s. Indeed, activated ILC2s have been shown to infiltrate the skin lesions of AD, both in humans and in a mouse model.⁹

However, TER from our in vitro KC-ILC2 coculture data indicates that ILC2s inhibit the formation of TJ during keratinocyte differentiation and maturation rather than promoting epithelial barrier breakdown by selectively disrupting already established TJs. To address this question, we analyzed the number of KCs 72 hours

after starting the KC-ILC2 coculture. The number of KC was comparable between us and ILC2 groups (Figure S5), suggesting that ILC2-derived cytokines do not affect the proliferation of KCs in these cocultures. Thus, further studies are needed to investigate the keratinocyte barrier function in association with human ILC2s.

Recently reported successful clinical studies with dupilumab targeting IL-4R α chain—a receptor, which is also used by IL-13—in AD emphasize the importance of the role of IL-13 on skin and mucosal barriers.¹⁰ The present findings suggest that IL-13 derived from ILC2s may decrease TJ barrier function in AD and provide important information on the clinical significance of TJ barrier disruption by ILC2s. The ability of ILC2-derived IL-13 might also be an important factor contributing to the role played by these cells in allergic skin diseases and to asthma.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

Dr Kazunari Sugita, Dr Can Altunbulakli, Dr Hideaki Morita, Dr Atsuko Sugita, Dr Terufumi Kubo, Dr Ryoko Kimura, Dr Hiroyuki Goto, Dr Osamu Yamamoto, Dr Beate Rückert, and Dr Mübeccel Akdis have nothing to disclose. Dr Cezmi A. Akdis: Relevant financial activities outside the submitted work: Consultancy; Actellion, Allergopharma, Circacia, Employment; Swiss Institute of Allergy and Asthma Research, University of Zurich, Switzerland, Grants/grants pending; Swiss National Science Foundation Research on T cell interaction with the epithelium, Allergopharma-Merck, Germany, CURE: European Commission's Seventh Framework Programme, Research on asthma, microbiome, and bacteriophages, Christine Kühne-Center for Allergy Research and Education Research on severe allergies, Other relationships; No other relationships/conditions/circumstances that present a potential conflict of interest

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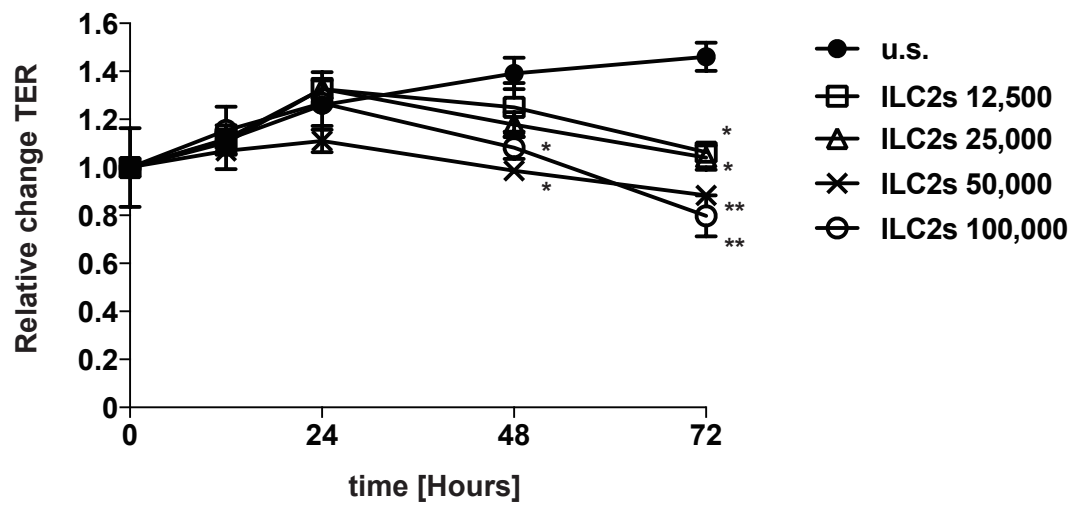


FIGURE S1

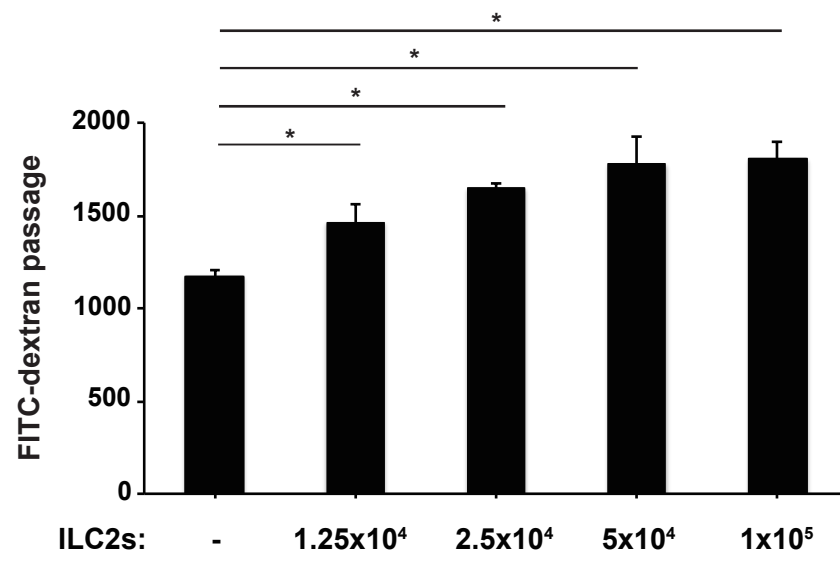


FIGURE S2

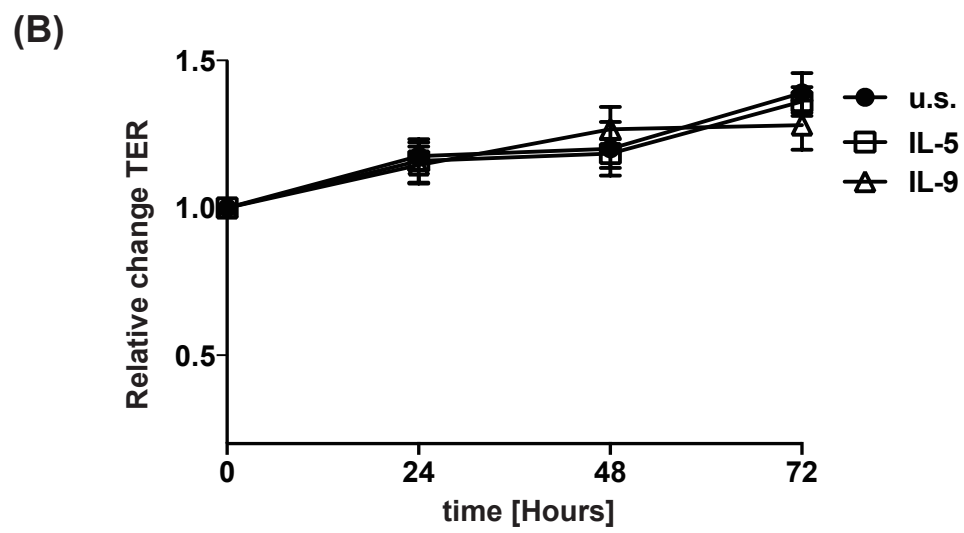
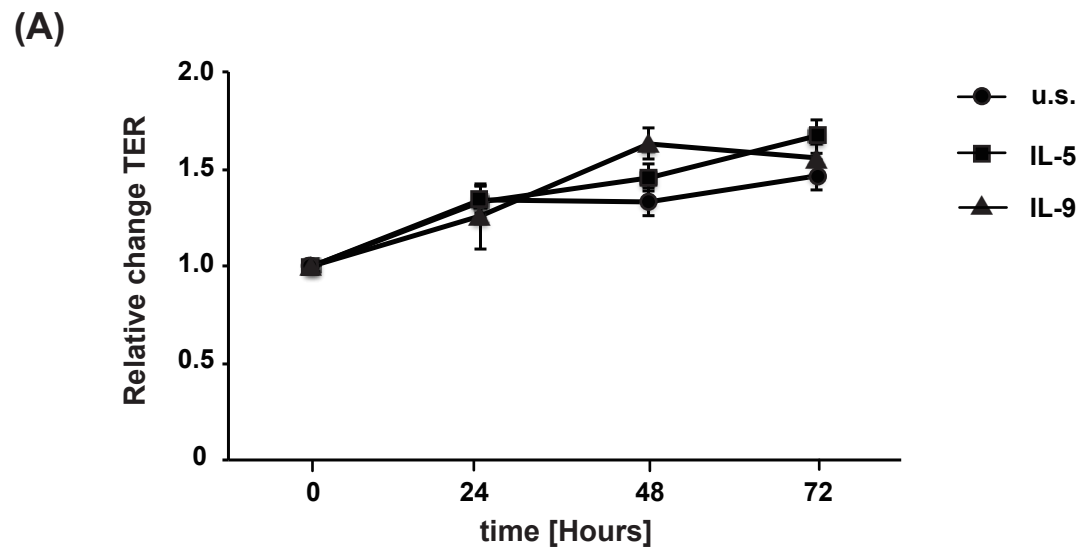


FIGURE S3

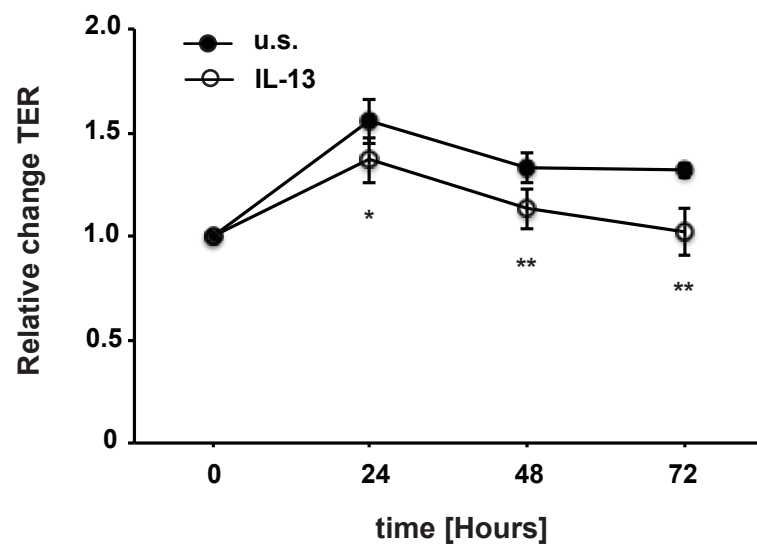


FIGURE S4

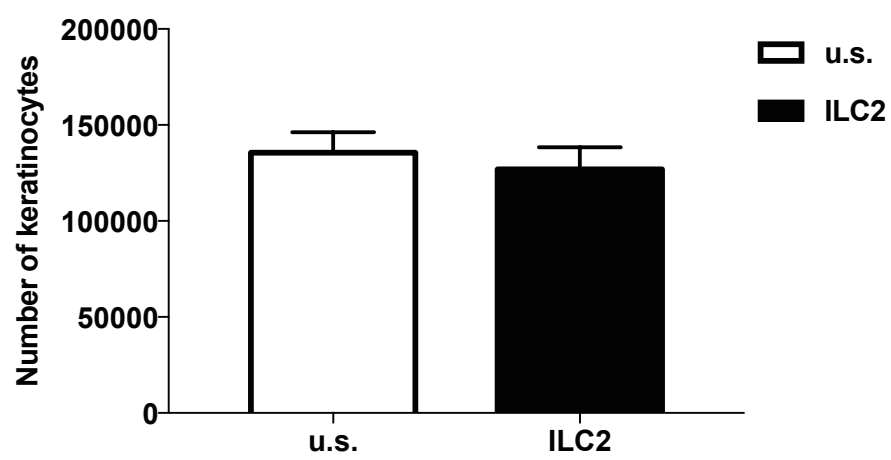


FIGURE S5

**Human type 2 innate lymphoid cells disrupt skin keratinocyte
tight junction barrier by IL-13**

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Online Supporting Information

NHEK culture, ILC2 sorting and ILC2 co-cultures in ALI

Primary human epidermal keratinocytes were purchased from PromoCell (Heidelberg, Germany). Blood from healthy individuals, for PBMC isolation was obtained from Red Cross, Switzerland. All experiments were conducted according to the Declaration of Helsinki and in accordance with the institutional ethical guidelines.

NHEKs from healthy subjects were seeded at a density of 110,000 cells in a 6.5-mm diameter polyester membrane with a pore size of 0.4 μ m (Corning, Corning, NY, USA) in medium for the ALI culture, as reported previously, to develop ALI cultures.^{1,2} Once the cells grew to complete confluence, medium in the apical compartment was removed to allow the cells to differentiate.

Human peripheral mononuclear cells (PBMCs) from four different donors were isolated by density gradient centrifugation Ficoll (Biochrom, Berlin, Germany). They were depleted of T cells, B cells and monocytes by labeling with a cocktail of antibodies against CD3, CD14 and CD19, followed by magnetic separation using an autoMACS Pro Separator. After separation of CD3⁻CD14⁻CD19⁻ cells, cells were stained with antibodies to lineage markers (CD1a, CD3, CD4, TCR $\alpha\beta$, TCR $\gamma\delta$, CD11c, CD14, CD16, CD19, CD34, CD56, CD94, CD123, CD303, and Fc ϵ R1 α) and antibodies to CD127, CD45 and CD294.

Lin⁻CD45⁺CD127⁺CRTH2⁺ ILC2s were sort-purified using a FACS Aria III cell sorter (BD

Biosciences, Franklin Lakes, NJ). Purity of sort-purified ILC2s was routinely greater than 99%. These cells were then expanded as reported previously.³

Then NHEKs and ILC2s were cultured together in the presence of transwell inserts, in which NHEKs were placed in the upper compartments and ILC2s were in the lower. In all experiments except the dose-response experiment, 100×10^3 of ILC2s were added to the basolateral compartments. In the dose-response experiment, increasing amounts of ILC2s (12.5 , 25 , 50×10^3 , and 100×10^3) were used per well. In some experiments, ILC2s were simultaneously cultured separately without NHEKs. Neutralizing experiments were performed using IL-13 blocking mAb (Source, polyclonal goat IgG; R&D Systems, Wiesbaden, Germany) which was used at a concentration of $10 \mu\text{g/ml}$, according to the manufacturer's protocol. Isotype control (R&D Systems) was used at the same concentration.

TER and paracellular flux measurements

TER and the paracellular diffusion of FITC-labeled 4 kDa dextran (Sigma-Aldrich, St Louis, MO) were measured as markers of barrier integrity. TER was measured at 0, 12, 24, 48, 72, and 96 hours using a Millicell-ERS Volt Ohm Meter (Millipore, Temecula, CA). Twenty-four hours after the apical addition of 2 mg/ml of FITC-dextran, paracellular permeability was assessed using an ELISA reader (Mithras LB 940; Berthold Technologies, Bad Wildbad, Germany) at 480 nm.

Immunofluorescence staining of tight junction

ALI cultured cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, St Louis, MO, USA) and permeabilized with PBS containing 0.1% Triton-X (Acros Organics, Geel, Belgium) and 0.02% SDS (Sigma-Aldrich). Specimens were then incubated with Alexa Fluor 488-labeled anti-occludin mAb (Invitrogen, Carlsbad, CA, USA) at room temperature

for 2 hours. Slides were then mounted with ProLong-Gold antifade reagent (Invitrogen). Specimens were examined under a confocal microscope (Leica TCS SPE, Heerbrugg, Switzerland). Data were analyzed and fluorescent intensities quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

mRNA isolation and quantitative PCR analysis

Total RNA was extracted and purified from ALI-cultured keratinocytes with an RNeasy Mini Kit (Qiagen, Hilden, Germany) and RNase-free DNase (Qiagen) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using a RevertAid RT kit containing random hexamers (Thermo Fisher Scientific, Waltham, MA, USA). Then, quantitative PCR was performed with target gene-specific primers (Microsynth, Balgach, Switzerland) and SYBR green PCR master mix (Thermo Fisher Scientific) on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. For calculation, the amount of elongation factor 1 α (EF1 α) mRNA was used to standardize the quantities of each transcript according to the $-2^{\Delta\Delta CT}$ method.⁴ The following primers were used: EF1 α , fw 5'-CTGAACCATCCAGGCCAAAT-3' rv 5'-GCCGTGTGGCAATCCAAT-3'; Claudin-1, fw 5'-CAGTCAATGCCAGGTACGAATTT-3' rv 5'-AAGTAGGGCACCTCCCAGAAG-3'; Claudin-4, fw 5'-TGTACCAACTGCCTGGAGGAT-3' rv 5'-GACACCGGCACTATCACCATAA-3'; Occludin, fw 5'-GAT GAGCAGCCCCCAAT-3' rv 5'-GGTGAAGGCACGTCCTGTGT-3'; Tricellulin, fw 5'-GCCCGATACCGGTTTCAGAGTCCT-3' rv 5'-TGATTTTCACACCTGCGCAGCC-3'; ZO-1, fw 5'-ACAGTGCCTAAAGCTATTCCTGTGA-3' rv 5'-TCGGGAATGGCTCCTTGAG-3'; E-cadherin, fw 5'-CCCACCACGTACAAGGGTCAGG-3' rv 5'-CAGCCTCCCACGCTGGGGTA-3'.

Quantification of cytokines

IL-4, IL-5, IL-9 and IL-13 levels in culture supernatants were examined using the MILLIPLEX Map Human Magnetic Bead Panel (Millipore, Billerica, MA, USA). Fluorescent signals were analyzed using the Bio-Plex 200 System (Bio-Rad, Hercules, CA, USA).

Figure legends

FIGURE S1 ILC2s reduced TER in a dose-dependent manner. NHEKs were incubated with the indicated ILC2s applied basolaterally, followed by TER measurements at different time points after the start of NEEK-ILC2 coculture. All data are expressed relative to TER measured in control cells. Data were obtained from two independent experiments performed in triplicates. $*P < .05$ and $**P < .01$, *u.s.*, unstimulated.

FIGURE S2 Effects of ILC2 dose on FITC-dextran passage. NHEKs were treated with ILC2s for 72 hours. ILC2s increased FITC-dextran penetration of ALI cultures of NHEKs. Data were obtained from three independent measurements in triplicates. $*P < .05$

FIGURE S3 Effects of IL-5 or IL-9 on ALI-cultured NHEKs. NHEKs were incubated with or without various cytokines, followed by TER measurements at different time points after cocultures. A, High doses of IL-5 (50 ng/ml) and IL-9 (50 ng/ml) are shown. B, TER using lower concentrations of IL-5 (5 ng/ml) and IL-9 (0.15 ng/ml) are shown. These doses are in range of secreted amounts. IL-5 or IL-9 does not modulate the barrier function both at high and small doses. Data are obtained from two independent experiments in quadruplicates

FIGURE S4 Effect of IL-13 (50 ng/ml) on ALI-cultured NHEKs. NHEKs were incubated with or without IL-13, followed by TER measurements at different time points after co-culturing. Data were expressed relative to TER measured in control cells. Analysis was done in triplicates, and data represent two independent experiments. * and ** indicate $P<.05$ and $P<.01$ respectively.

FIGURE S5 The number of ALI-cultured NHEKs with ILC2s for 72 h. To detach cells from the culture dish, 100 μ l and 500 μ l of 0.05% Trypsin-EDTA (Thermo Fisher Scientific, MA, USA) was added to the apical and basolateral compartments of the transwell insert, respectively. Incubated at 37 degrees for 10 minutes. Then, collected the trypsinized cells into 1.5 ml tube and added 600 μ l of trypsin neutralizing solution (Thermo Fisher Scientific, MA, USA). The number of keratinocytes was calculated using hemacytometer. Analysis was performed in quadruplicates, and data represent two independent experiments..

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