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The Loss of H3K27 Histone Demethylase Utx in T Cells Aggravates Allergic Contact Dermatitis

Takashi Inoue,^{*,1} Miyuki Omori-Miyake,^{†,1} Saho Maruyama,[‡] Masataka Okabe,[§] Makoto Kuwahara,[‡] Hiroaki Honda,[¶] Hiromasa Miura,^{*} and Masakatsu Yamashita^{†,‡}

The pathogenesis of allergic contact dermatitis (ACD) requires the activation of Ag-specific T cells, including effector and regulatory T cells. The differentiation and function of these T cells is epigenetically regulated through DNA methylation and histone modifications. However, the roles of altered histone H3K27 methylation in T cells in the development of ACD remain unknown. Two types of histone H3K27 demethylases, Utx and Jmjd3, have been reported in mammals. To determine the role of the histone H3K27 demethylase expression of T cells in the development of ACD, we generated T cell-specific, *Utx*-deficient (*Utx* KO) mice or *Jmjd3*-deficient (*Jmjd3* KO) mice. Unlike control mice, *Utx* KO mice had severer symptoms of ACD, whereas *Jmjd3* KO mice showed symptoms identical to those in control mice. In *Utx* KO mice with ACD, the massive infiltration of myeloid cells, including neutrophils and dendritic cells, has been observed. In addition, the expression of proinflammatory cytokines in CD4⁺ T cells of the draining lymph nodes (LNs) and in CD8⁺ T cells of the skin was increased in *Utx* KO mice, whereas the ratio of Foxp3⁺ regulatory CD4⁺ T cells to Foxp3[−] conventional CD4⁺ T cells was decreased in both the draining LNs and the skin of *Utx* KO mice with ACD. Furthermore, Foxp3⁺ regulatory CD4⁺ T cells of *Utx* KO mice with ACD expressed a decreased level of CCR4 (a skin-tropic chemokine receptor) in comparison with control. Thus, in CD4⁺ T cells, *Utx* could potentially be involved in the regulation of the pathogenesis of ACD. *The Journal of Immunology*, 2021, 207: 1–12.

It has been well established in research that allergic contact dermatitis (ACD) is induced by exposure to environmental allergens. The prevalence of ACD in general individuals is ~20.1% in adults and 16.5% in nonadults, with a female predominance (1). Because contact allergens exist in very common products used in daily life, as well as in industrial and occupational settings (2, 3), it is difficult to remove potential allergens from daily life and prevent the onset of ACD in association with allergic inflammation.

Allergic inflammation after exposure to a certain contact allergen is caused by delayed-type hypersensitivity reactions through Ag-specific T cells (3). Ag-specific effector T cells contribute to the development of the pathogenesis of ACD by producing proinflammatory cytokines and chemokines. In addition, Ag-specific regulatory T (T_{reg}) cells are involved in the regulation of inflammation with their anti-inflammatory functions (3, 4).

Epigenetic modifications such as histone modifications have effects on the functions of immune cells (e.g., T cells and macrophages) and other participants (e.g., epidermal keratinocytes and dermal fibroblasts) that are associated with the development of allergic inflammation (5, 6). In addition, the responses of Ag-specific T

cells, including the functional differentiation and the phenotypic plasticity of each subset of T cells, are tightly controlled by the gene expression through epigenetic modifications (7–9). Among the modifications of histones, the methylation mark of H3K27 represents a repressive transcription, whereas the methylation mark of H3K4 is a hallmark of an active transcription (10, 11). Thus, the modifications of the methylation of H3K4 and H3K27 are involved in the determination of the cell fate and function of CD4⁺ T cells (12).

Ezh2, Utx, and Jmjd3 regulate the methylation status of histone H3K27 (13, 14). Ezh2 methylates the residue of histone H3K27, whereas Utx and Jmjd3 demethylate the di- or trimethylated H3K27 (15). Utx has a role in promoting the differentiation of CD4⁺ T cells into Th2 cells (M. Kuwahara, M. Izumoto, H. Honda, K. Inoue, Y. Imai, J. Suzuki, S. Maruyama, M. Yasukawa, and M. Yamashita, manuscript posted on bioRxiv, DOI: 10.1101/184648). However, Jmjd3 has roles in promoting the differentiation of CD4⁺ T cells into Th1 cells and in suppressing differentiation into Th17 cells (16). Two studies reporting the function of Jmjd3 in the differentiation into Th2 cells have reported conflicting results (M. Kuwahara, et al., manuscript posted on bioRxiv, DOI: 10.1101/184648).

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M.Y. wrote the paper; M.O.-M. and M.Y. provided critical discussions and edited the manuscript; H.M. provided critical discussions; H.M. and M.Y. supervised the project. All authors approved the final version of the manuscript.

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Abbreviations used in this article: ACD, allergic contact dermatitis; ChIP, chromatin immunoprecipitation; CM, central memory; DC, dendritic cell; DNFB, 2,4-dinitro-1-fluorobenzene; EM, effector memory; iTreg, inducible regulatory T; LN, lymph node; PoAb, polyclonal Ab; rm, recombinant murine; RM, resident memory; T_{CM}, central memory T; T_{conv}, conventional T; T_{EM}, effector memory T; T_{reg}, regulatory T; T_{RM}, tissue-resident memory T.

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(16). Furthermore, *Jmjd3* is involved in the peripheral differentiation of CD4⁺ T cells into T_{reg} cells. Although functional roles of *Utx* and *Jmjd3* in the differentiation of T cells into effector cells remain partially unknown, both the studies consistently showed that *Utx* and *Jmjd3* could be involved in the differentiation of T cells into effector cells. Most important, the role of *Utx* and *Jmjd3* in the pathogenesis of T cell-mediated immune disorders remains to be elucidated.

In this study, we examined the effects of either *Utx* deficiency or *Jmjd3* deficiency in T cells on the pathogenesis of ACD. As a result, unlike *Jmjd3* deficiency in T cells, the *Utx* deficiency aggravated the symptoms of ACD. The cellular and histological analyses of the skin lesions and the draining lymph nodes (LNs) showed that *Utx* deficiency in T cells increased the production of proinflammatory cytokines in the skin lesions or the draining LNs but decreased the ratio of T_{reg} cells to conventional CD4⁺ T (T_{conv}) cells in skin lesions and the draining LNs. In addition, *Utx* deficiency in CD4⁺ T cells modified their differentiation into effector cells and T_{reg} cells. We thus concluded that in T cells, *Utx* but not *Jmjd3* negatively regulates the pathogenesis of ACD.

Materials and Methods

Mice

T cell-specific, *Utx*-deficient mice and *Jmjd3*-deficient mice have been established by crossing Cre recombinase transgenic mice under the control of the *Cd4* promoter (*Cd4-Cre* Tg mice) with either *Utx*^{flox/flox} mice or *Jmjd3*^{flox/flox} mice. The *Cd4-Cre* Tg mice were purchased from the BioResource Research Center at the RIKEN Institute, Japan. *Utx*^{flox/flox} mice (17) and *Jmjd3*^{flox/flox} mice (18) were established by Drs. Kazuki Inoue and Yuki Imai (Ehime University) and Dr. Hiroaki Honda (Tokyo Women's Medical University), respectively. C57BL/6 mice that were purchased from Clea Japan (Tokyo, Japan) were used as control mice after the mice became acclimatized to the environment of the animal facility at least for 1 wk. All mice at 8–13 wk of age with a C57BL/6 background were maintained under specific pathogen-free conditions in the animal facility at Ehime University Graduate School of Medicine. All experiments were performed on either female or male mice, except for *Utx*^{flox/flox} mice crossed with *Cd4-Cre* Tg mice, for which experiments were performed on female mice. All the animal procedures were approved by the Ehime University Animal Care and Use Committee and the Gene Recombination Experiment Safety Committee of Ehime University Graduate School of Medicine.

ACD models

The method of inducing ACD in our present study was established on the basis of previously described methods (19). Mice were anesthetized, and then 100 μ l of 0.15% 2,4-dinitro-1-fluorobenzene (DNFB; Sigma-Aldrich, Tokyo, Japan) dissolved in acetone/olive oil (3:1) was applied on the surface of their shaved abdomen. Five days after the first application of 0.15% DNFB solution, either 25 μ l of 0.15% DNFB solution was applied to each side of the auricles (the auricle skin model) or 50 μ l of 0.15% DNFB solution was applied onto the dorsal skin of the lumbar area that was shaved with hair clippers (the back skin model) three times with 5-d intervals. In some experiments, acetone/olive oil was applied instead of 0.15% DNFB solution as a control. The ear thickness was measured at the indicated durations using a thickness gauge (Dial thickness gauge; Mitsutoyo, Tokyo, Japan). The increase in ear thickness versus baseline was calculated by subtraction.

Histology

Auricle skin samples were taken 24 h after the final application of DNFB, fixed in 4% paraformaldehyde (Thermo Fisher Scientific, Tokyo, Japan), embedded in paraffin, sectioned at 5 μ m, and stained with H&E. Images were photographed using a BZ-9000 digital microscope (Keyence Co., Osaka, Japan).

Preparation and enrichment of cells from the draining LNs, auricle, or spleen

For the preparation of cells from the draining LNs, one pair of axillary, brachial, and inguinal LNs were pooled. For the preparation of cells from the auricle skin, pieces of the auricle skin were pooled, minced, and digested with 0.13 U/ml Liberase Thermolysin Medium (Sigma-Aldrich) and 50 U/ml DNase I (Nippon Gene, Tokyo, Japan) for 45 min at 37°C. Six pieces of

auricle from wild-type (WT) mice and four pieces of auricle from *Utx* KO mice were used for the analysis of the granulocytes or other innate cells. Six pieces of auricle from WT or *Utx* KO mice were used for flow cytometry of T cells. Total CD4⁺ T cells from LNs for ex vivo analysis were positively selected using anti-CD4-FITC with anti-FITC Microbeads Ultrapure (Miltenyi Biotec, Tokyo, Japan) on an autoMACS separator at the purity of >95%. Total CD4⁺ T cells from spleen for in vitro culture were negatively selected using a MojoSort Mouse CD4 T Cell Isolation Kit (BioLegend, Tokyo, Japan) at >95% purity. CD4⁺CD44^{lo}CD62L^{hi} naive CD4⁺ T cells from spleen for in vitro culture were negatively selected using a MojoSort Mouse CD4 T Cell Isolation Kit in combination with biotinylated anti-mouse/antihuman CD44 mAb (clone IM7; BioLegend) and biotinylated anti-mouse CD25 mAb (clone PC61; BioLegend) on an autoMACS separator at the purity of >95%.

Flow cytometry and sorting

Whole or enriched single cells were stained with Abs. The following mAbs were used: anti-CD3 (clone 145-2C11), anti-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-CD11b (clone M1/70), anti-CD11c (clone N418), anti-CD19 (clone eBio 1D3), anti-CD44 (clone IM7), anti-CD45 (clone 30-F11), anti-CD45R (clone RA3-6B2), anti-CD49b (clone DX5), anti-CD62L (clone MEL-14), anti-CD64 (clone X54-5/7.1), anti-CD69 (clone [1H]2F3), anti-CD103 (clone 2E7), anti-Foxp3 (clone 150D), anti-IA/IE (clone M5/114.15.2), anti-IFN- γ (clone HMG1.2), anti-IL-2 (clone JES6-5H4), anti-IL-4 (clone 11B11), anti-IL-17A (clone TC11-18H10.1), anti-IL-17F (clone 9D3.1C8), anti-Ly6G (clone 1A8), anti-Siglec-F (clone E50-2440), anti-TCR- β (clone H57-597), and anti-TER119 (clone TER-119) (purchased from BioLegend, BD Biosciences, eBioscience, Thermo Fisher Scientific, or TONBO Biosciences, all located in Tokyo, Japan). The following fluorescent conjugations of Abs were used: FITC, PE, PE—cyanine 7, allophycocyanin, Alexa Fluor 647, allophycocyanin—cyanine 7, Brilliant Violet 421, Violet 450, or Brilliant Violet 510. For the staining of lineage markers, biotinylated Abs were used in combination with Brilliant Violet 605 streptavidin. For the staining of cells in the auricle skin, the cells were treated with anti-CD16/32 (clone 93; BioLegend) before the staining with fluorescent conjugated Abs. Stained cells were analyzed on a Gallios cytometer with the Kaluza software program (Beckman Coulter, Tokyo, Japan), the CytoFLEX cytometer with the CytExpert 1.0 software program (Beckman Coulter), or a FACSARIA II cell sorter with the FACSDiva software program (Beckton Dickinson, Tokyo, Japan). Cell sorting was performed using a BD FACSARIA II cell sorter with the FACSDiva software program at the purity of >98%. All data were analyzed using the FlowJo software program, version 10.4.2 (BD Biosciences, Ashland, OR). The gating strategy was designed on the basis of a previous study (20).

In vitro culture of CD4⁺ T cells

CD4⁺ T cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mmol/l nonessential amino acids, 10 mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin, 55 μ M 2-ME, and 10% FBS in a humidified atmosphere of 5% CO₂ at 37°C. The cells were stimulated with immobilized anti-TCR- β mAb (10 μ g/ml, clone H57-597; BioLegend) and soluble anti-CD28 mAb (1 μ g/ml, clone 37.5; BioLegend) for the first 2 d and expanded for 5 d (ThN, Th1, and Th2 conditions), 3 d (Th17 and inducible Treg [iTreg] conditions), or the indicated periods. The following culture conditions were used: ThN condition, 10 ng/ml recombinant murine (rm)IL-2 (TONBO Biosciences); Th1 culture condition, 10 ng/ml rmIL-2, 0.1 ng/ml rmIL-12 (PeproTech, Rocky Hill, NJ), and 2.5 μ g/ml anti-IL-4 mAb (clone 11B11; BioLegend); Th2 culture condition, 10 ng/ml rmIL-2, 1 ng/ml rmIL-4 (PeproTech), and 2.5 μ g/ml anti-IFN- γ mAb (clone R4-6A2; BioLegend); Th17 culture condition, 10 ng/ml rmIL-1 β (FUJIFILM Wako, Osaka, Japan), 5 ng/ml rmIL-6 (Fujifilm Wako), 1 ng/ml recombinant human TGF- β (R&D Systems, Minneapolis, MN), 2.5 μ g/ml anti-IFN- γ mAb, 2.5 μ g/ml anti-IL-4 mAb, and 2.5 μ g/ml anti-IL-2 mAb (clone JES6-1A12; BioLegend); iTreg condition, 10 ng/ml rmIL-2, 1 ng/ml recombinant human TGF- β , 2.5 μ g/ml anti-IFN- γ mAb, and 2.5 μ g/ml anti-IL-4 mAb.

Cytokine measurements

For intracellular cytokine staining, the cultured cells were restimulated with immobilized 10 μ g/ml anti-TCR- β mAb for 6 h in the presence of 2 μ M monensin. For intracellular cytokine staining, cells were fixed in 4% paraformaldehyde, permeabilized, blocked, and stained with the indicated mAbs. Cytokine production was analyzed on a flow cytometer. For ELISA, the cells were restimulated with immobilized 10 μ g/ml anti-TCR- β mAb for 24 h. The cytokines contained in the restimulated culture supernatant were

measured by a sandwich ELISA using an optimal pair of Abs as follows: IFN- γ , clone R4-6A2 and clone XMG1.2; IL-2, clone JES6-1A12 and clone JES6-5H4; IL-4, clone BVD4-1D11 and clone BVD6-24G2; and IL-5, clone TRFK5 and clone TRFK4 (BD Biosciences). ELISAs for IL-10 and GM-CSF were performed with Ready-SET-Go! ELISA (Thermo Fisher Scientific) according to the manufacturer's protocols. The ELISAs for IL-17, IL-17F, and IL-13 were performed with the DuoSet ELISA (R&D Systems) according to the manufacturer's protocols.

Quantitative RT-PCR

For ex vivo analysis, cells were stimulated with or without 10 μ g/ml anti-TCR- β for 4 h, then lysed in TRI reagent (Molecular Research Center, Cincinnati, OH). For in vitro analysis, cultured cells were harvested and lysed in TRI reagent. Total RNA was extracted. cDNA was synthesized using SuperScript VILO reverse transcriptase (Invitrogen/Thermo Fisher Scientific). cDNA of stimulated cells was used as a template for quantitative PCRs for the detection of cytokine genes, whereas cDNA of unstimulated cells was used for the detection of other genes. The expression of genes was detected with specific primers and Roche Universal Probes (Nippon Gene). The primers and probes used were as follows: *18s* rRNA: 5'-GCAAT-TATCCCCATGAACG-3' (forward), 5'-GGGACTTAATCAACGCAAGC-3' (reverse), probe 48; *Ccr4*: 5'-CTCAGGATCACTTCAGAGCA-3' (forward), 5'-GGCATTTCATTTGGAATCG-3' (reverse), probe 18; *Ccr8*: 5'-AG-AAGAAAGGCTCGCTCAGA-3' (forward), 5'-GGCTCCATCGTGAATC-CAT-3' (reverse), probe 4; *Ccr10*: 5'-TGGCAATGGCTAGTCTTG-3' (forward), 5'-AGGTGGGAGATCGGGTAGTT-3' (reverse), probe 71; *Csf2*: 5'-GCATGTAGAGGCCATCAAGA-3' (forward), 5'-CGGGTCTGCACACA-TGTTA-3' (reverse), probe 79; *Foxp3*: 5'-TCAGGAGCCACAGTACA-3' (forward), 5'-TCTGAAGCAGAGTCAGGAGA-3' (reverse), probe 78; *Gata3*: 5'-TTATCAAGCCCAAGCGAAG-3' (forward), 5'-TGGTGGTGGTCTGACA-GTT-3' (reverse), probe 108; *Ifng*: 5'-ATCTGGAGGAAGTGGCAAAA-3' (forward), 5'-TTCAAGACTTCAAGAGTCTGAGGTA-3' (reverse), probe 21; *Il4*: 5'-GATCGGCATTTTCAACGAG-3' (forward), 5'-CGAGTCACTCTCTGTG-TTG-3' (reverse), probe 92; *Il10*: 5'-CAGAGCCACATGCTCTAGA-3' (forward), 5'-TGTCAGCTGGTCTTTGTT-3' (reverse), probe 41; *Il13*: 5'-ACCC-AGAGGATATTGC-3' (forward), 5'-TGGGTACTTTCGATTT-3' (reverse), probe 75; *Il17f*: 5'-CCCAGGAAGACATACT-3' (forward), 5'-CAACAG-TAGCAAGAC-3' (reverse), probe 46; *Il22*: 5'-TTTCTGACCAACT-CAGCA-3' (forward), 5'-TCTGGATGTTCTGGTCGTCA-3' (reverse), probe 17; *Ilgae*: 5'-GCAGAGAACCACAGGACGA-3' (forward), 5'-CAATGAT-GAGAGGCAAGAGC-3' (reverse), probe 63; *Kdm6a*: 5'-CGGGTTCGTG-AGGTTTCAT-3' (forward), 5'-GAGATTCTGTAGCAGCAACA-3' (reverse), probe 3; *Kdm6b*: 5'-GTACAGACCCCGGAACC-3' (forward), 5'-TGGT-GGAGAAAAGGCCTAAG-3' (reverse), probe 20; *Rorc*: 5'-CAGCGCAC-CAACCTCTTT-3' (forward), 5'-CCCACATCTCCACATTGA-3' (reverse), probe 52; *Thx21*: 5'-TCAACAGCACCAGACAGAG-3' (forward), 5'-AAA-CATCTGTAAATGGCTTGTG-3' (reverse), probe 19; *Tnfr*: 5'-TCTTCTC-ATTCTGCTGTGG-3' (forward), 5'-GGTCTGGCCATAGAAGTGA-3' (reverse), probe 49; *Kdm6a* (exon 2, for deleted region in *Utx* KO): 5'-CGGGTTCGTGAGGTTTCAT-3' (forward), 5'-GAGATTCGTAGCAGCGA-ACA-3' (reverse), probe 3; *Kdm6b*: 5'-GTACAGACCCCGGAACC-3' (forward), 5'-TGGTGGAGAAAAGGCCTAAG-3' (reverse), probe 20; *Kdm6b* (exon 17, for deleted region in *Jmjd3* KO): 5'-GGGTAACATCCACA-GGCAAC-3' (forward), 5'-TGTATAGCTGCACGGTGTTCAT-3' (reverse), probe 102. For the detection of *Il5* (Mm00439646_m1) and *Il17a* (Mm004396180_m1) genes, predeveloped assay reagent kits of primers and probes (TaqMan; Thermo Fisher Scientific) were used.

Western blotting

Cells were washed with ice-cold PBS, and nuclear and cytoplasmic lysates were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). The lysates were denatured, separated with SDS-PAGE on a 10% acrylamide gel, and transferred onto a polyvinylidene difluoride membrane. The blots were probed with rabbit anti-UTX mAb (clone D3Q11), rabbit anti- β -actin mAb (clone 13E5), rabbit anti-H3K27me2 polyclonal Ab (PoAb) (39245), rabbit anti-H3K27me3 PoAb (39155), and mouse anti-histone H3 mAb (39763) (from either Cell Signaling Technology, Tokyo, Japan, or Active Motif, Carlsbad, CA). The immunoreactive proteins were visualized with peroxidase-conjugated antirabbit IgG (H+L) or antigoat IgG (H+L) (Jackson ImmunoResearch Laboratory, West Grove, PA) using an ECL detection system (GE Healthcare, Tokyo, Japan). The images were captured using a ChemiDoc XRS Plus luminescent image analyzer (Bio-Rad Laboratories, Hercules, CA).

Chromatin immunoprecipitation (ChIP)-quantitative PCR

The ChIP assay was performed using the Magna ChIP G kit (17-611; Merck Millipore, Burlington, MA) according to the manufacturer's protocol.

Briefly, 1 million cells per one ChIP reaction of freshly isolated CD4⁺ T cells or CD4⁺ T cells that were cultured for 5 d under one ThN condition were cross-linked with 1% paraformaldehyde in PBS and sonicated. The sheared chromatin lysates were precipitated with 2 μ g of rabbit PoAb anti-histone H3K27me2 (39245; Active Motif) or 5 μ g of rabbit PoAb anti-histone H3K27me3 (39155; Active Motif) Ab. The precipitated genome was amplified with gene-specific primers and Roche Universal Probes (Nippon Gene). The primers and probes used were as follows: *Ifng* promoter region (*Ifng-p2*): 5'-GAGGAGCCTTCGATCAGGTA-3' (forward), 5'-TTCTCTAG-GTCAGCCGATGG-3' (reverse), probe 106; *Csf2* promoter region: 5'-GCA-GTGAGCCCACTACTCAGA-3' (forward), 5'-CACAAATGCCAGGAAA-AGTAA-3' (reverse), probe 5; *Foxp3* exon 1 region: 5'-CATCTCTCC-ATCCAAGCTC-3' (forward), 5'-CTTCCAAGTCTCGTCTGAAGG-3' (reverse), probe 2.

Statistical analyses

All statistical analyses were performed using the Prism software program (version 6 or version 9; GraphPad Software, La Jolla, CA). Differences between the two groups were tested using two-tailed unpaired *t* tests. Differences among multiple groups were tested using either one-way or two-way ANOVA tests. The statistical significance is shown in the figures (**p* < 0.05, ***p* < 0.01, ****p* < 0.005, and *****p* < 0.001).

Results

Deficiency of *Utx*, but not *Jmjd3*, in T cells aggravated the skin symptoms of DNFB-derived ACD

Ag-specific CD4⁺ and CD8⁺ T cells (21) are involved in the development of ACD. Both *Utx* and *Jmjd3* play an important role in the differentiation and function of CD4⁺ and CD8⁺ T cells (M. Kuwahara, et al., manuscript posted on bioRxiv, DOI: 10.1101/184648) (16, 22). Thus, we first assessed the impact of the loss of *Utx* or *Jmjd3* in T cells upon the induction of ACD using T cell-specific *Utx* KO mice and *Jmjd3* KO mice. Of note, the mRNA expression of *Kdm6a* coding *Utx* and *Kdm6b* coding *Jmjd3* was definitely deleted in CD4⁺ T cells of *Utx* KO mice and *Jmjd3* KO mice, respectively (Supplemental Fig. 1A). In addition, we confirmed the depletion of *Utx* protein in cultured *Utx* KO CD4⁺ T cells by immunoblotting (Supplemental Fig. 1B). However, the level of histone H3K27me2 and H3K27me3 was almost identical among WT, *Utx* KO, and *Jmjd3* KO CD4⁺ T cells (Supplemental Fig. 1C). Repeated applications of a hapten DNFB resulted in scaly and swollen skin in *Utx* KO mice in comparison with WT mice (Fig. 1A). In contrast, visual observation and the measurement of the ear thickness revealed no difference in the skin of *Jmjd3* KO mice and WT mice (Fig. 1B).

In order to evaluate the morphological differences in the skin, we analyzed histological sections of skin specimens, as shown in Fig. 1C and 1D. In comparison with auricle skin that was treated with vehicle (a mixture of acetone and olive oil), the skin treated with DNFB showed epidermal hyperplasia accompanied by mitotic figures in the epidermal keratinocytes, hyperkeratosis, hypertrophy of keratinocytes in the spinous and granular layers, and an increase in the intracellular space in the epidermis. In addition, cellular infiltration, including granulocyte infiltration and an increase in the number of fibroblasts, was observed in the dermis of WT mice that were treated with DNFB. DNFB-treated *Utx* KO mice had epidermal hyperplasia and increased mitotic figures, a decrease in granular layer keratinocytes, cellular hypertrophy in spinous layer keratinocytes, and microabscesses and massive infiltration of cells into the epidermis, dermis, and s.c. tissue. On the one hand, the junction between the dermis and s.c. tissue was not clear in the skin of *Utx* KO mice that received DNFB (Fig. 1C). On the other hand, *Jmjd3* KO mice that were treated with DNFB had epidermal hyperplasia along with hypertrophy of keratinocytes and larger intracellular spaces in the spinous layer of keratinocytes, whereas the intensity of symptoms of *Jmjd3* KO mice was milder than the symptoms of *Utx*

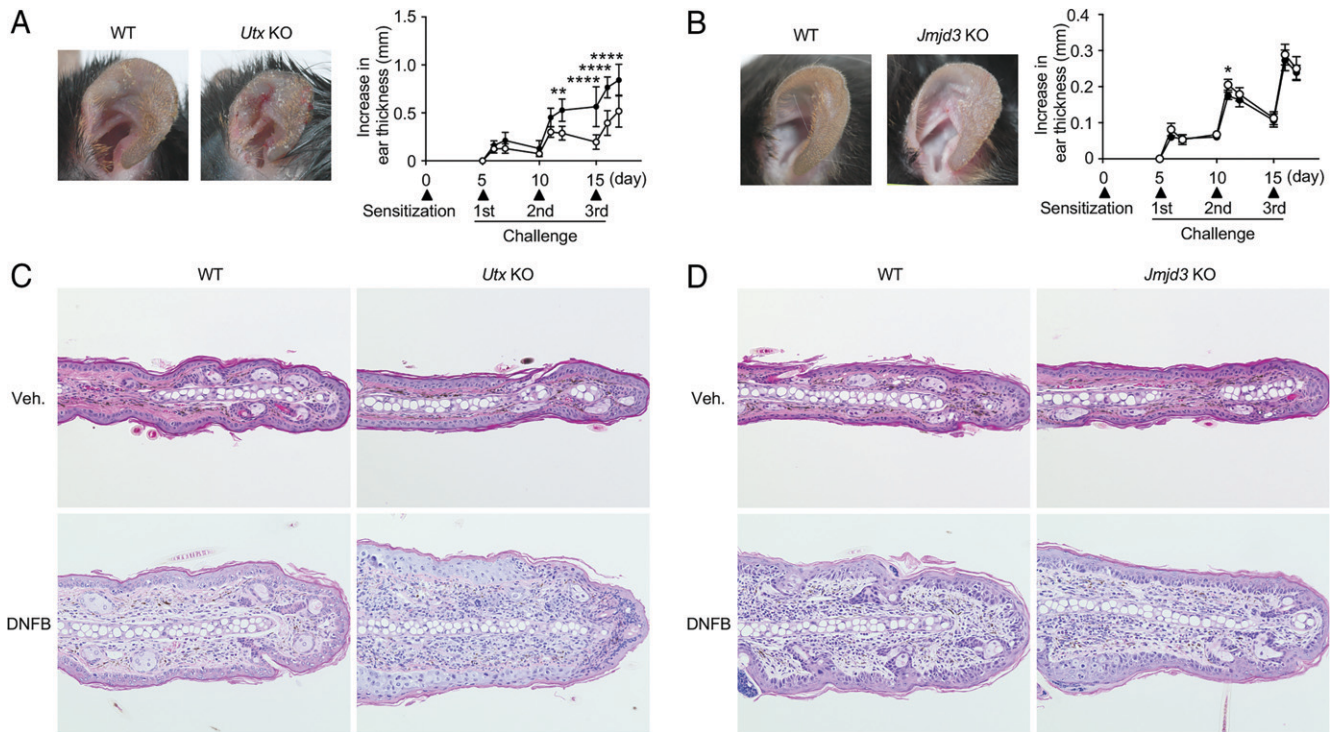


FIGURE 1. Skin inflammation was aggravated in *Utx* KO mice but not in *Jmjd3* KO mice. Representative images (left) and changes in the ear thickness (right) of *Utx* KO mice (**A**) and *Jmjd3* KO mice (**B**). Plots and error bars indicate the mean and SD, respectively. (**A**) WT (open circles; $n = 5$) and *Utx* KO (filled circles; $n = 5$). (**B**) WT (open circles; $n = 6$) and *Jmjd3* KO (filled circles; $n = 6$). (**C** and **D**) Representative images of auricle sections stained with H&E. Original magnification $\times 20$. (**C**) WT mice versus *Utx* KO mice. (**D**) WT mice versus *Jmjd3* KO mice. Two-way ANOVA followed by post hoc test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. DNFB, DNFB dissolved in acetone/olive oil; vehicle, acetone/olive oil; WT, wild type mouse as a control.

KO mice (Fig. 1D). No inflammation was observed in the auricle of either *Jmjd3* KO mice or WT mice that were treated with vehicle (Fig. 1C and 1D). As described below, this morphological analysis led us to focus on the role of the *Utx* expression in T cells in the development of DNFB-derived ACD.

Changes of immune cells in the lesional skin of *Utx* KO mice after repeated applications of DNFB

Upon repeated exposure to a hapten, inflammatory cells, including granulocytes, macrophages, and Ag-specific effector T cells, infiltrated the lesional skin (4). In order to compare the composition of immune cells that accumulated in ACD skin between WT mice and *Utx* KO mice, we established gating strategies for granulocytes (Supplemental Fig. 2A) and for monocytes, macrophages, and dendritic cells (DCs) (Supplemental Fig. 2B) and analyzed immune cells in the skin at 24 h after a third challenge with DNFB (Fig. 2A–2C). Consistent with the histological observations (Fig. 1C and 1D), the frequencies and cell numbers of granulocytes, neutrophils, and eosinophils, per auricle, were all increased in *Utx* KO mice: neutrophils expressing Ly6G (a neutrophil marker) (23) as a $CD45^{+}$ lineage marker (Lin^{-} $TCR-\beta^{-}$ $CD11b^{+}$ $Ly6G^{+}$, WT versus *Utx* KO, $53.2 \pm 4.0\%$ versus $50.2 \pm 9.8\%$ [$52.7 \pm 3.6 \times 10^3$ cells versus $152.7 \pm 30.2 \times 10^3$ cells], a 2.9-fold increase in the mean cell number; eosinophils expressing Siglec-F (an eosinophil marker) (24) as $CD45^{+}$ Lin^{-} $TCR-\beta^{-}$ $Ly6G^{-}$ IA/IE^{-} $CD11b^{+}$ $Siglec-F^{+}$, WT versus *Utx* KO, $23.5 \pm 15.5\%$ versus $60.4 \pm 13.7\%$ [$9.7 \pm 4.4 \times 10^3$ cells versus $96.5 \pm 57.7 \times 10^3$ cells], a 9.9-fold increase in the mean cell number (Fig. 2A and 2D).

In contrast, the cell numbers of monocytes in DNFB-treated *Utx* KO mice were slightly increased compared with those in the DNFB-treated WT mice: monocytes expressing CD64 (a monocyte/macrophage marker) as $CD45^{+}$ Lin^{-} $Ly6G^{-}$ IA/IE^{-} $SSC-A^{lo}$

$CD11b^{+}$ $CD64^{+}$, WT versus *Utx* KO, $46.9 \pm 24.9\%$ versus $52.3 \pm 27.1\%$ [$8.3 \pm 5.8 \times 10^3$ cells versus $11.2 \pm 13.3 \times 10^3$ cells], a 1.3-fold increase in the mean cell number. Only the cell numbers of macrophages and DCs were increased in *Utx* KO mice: macrophages as $CD45^{+}$ Lin^{-} $Ly6G^{-}$ IA/IE^{+hi} $CD11b^{+hi}$ $CD64^{+}$, WT versus *Utx* KO, $50.3 \pm 24.4\%$ versus $27.2 \pm 12.7\%$ [$14.3 \pm 10.2 \times 10^3$ cells versus $42.3 \pm 42.0 \times 10^3$ cells], a 3.0-fold increase in the mean cell number; DCs as $CD45^{+}$ Lin^{-} $Ly6G^{-}$ IA/IE^{hi} $CD11b^{-/+}$ $CD11c^{+}$ $CD64^{+}$, WT versus *Utx* KO, $16.1 \pm 3.1\%$ versus $4.2 \pm 1.8\%$ [$1.9 \pm 0.7 \times 10^3$ cells versus $3.4 \pm 0.4 \times 10^3$ cells], 1.8-fold increase as the mean cell number (Fig. 2B and 2E). Of note, the changes in the numbers of cells in *Utx* KO mice showed identical trends between experiments. Only the changes in neutrophils and DCs showed large statistically significant variations in the analysis (Fig. 2D–2F). The cell numbers of total $\alpha\beta$ T cells as $CD45^{+}$ $TCR-\beta^{+}$ $SSC-A^{lo}$, $CD4^{+}$ T cells as $CD45^{+}$ $TCR-\beta^{+}$ $SSC-A^{lo}$ $CD4^{+}$, and $CD8^{+}$ T cells as $CD45^{+}$ $TCR-\beta^{+}$ $SSC-A^{lo}$ $CD8^{+}$ were almost identical between *Utx* KO mice and WT mice, although the frequency varied: $CD4^{+}$ T cells, WT versus *Utx* KO, $30.5 \pm 7.6\%$ versus $21.7 \pm 4.2\%$ [$5.5 \pm 2.5 \times 10^3$ cells versus $5.3 \pm 2.0 \times 10^3$ cells]; $CD8^{+}$ T cells, WT versus *Utx* KO, $51.4 \pm 12.8\%$ versus $47.6 \pm 6.9\%$ [$11.3 \pm 9.1 \times 10^3$ cells versus $12.1 \pm 5.3 \times 10^3$ cells] (Fig. 2C and 2F).

In response to repeated Ag exposures, effector memory T (T_{EM}) cells and tissue-resident memory T (T_{RM}) cells accumulate in local sites of nonlymphoid tissues, such as the skin exposed to Ag rechallenge (25). In order to classify the type of memory T cells in the skin, we analyzed the expression profiles of surface markers. The frequency of $CD4^{+}$ T_{EM} cells ($CD45^{+}$ Lin^{-} $TCR-\beta^{+}$ $CD4^{+}$ $CD62L^{lo}$ $CD44^{hi}$) in the skin of DNFB-treated WT and *Utx* KO mice was identical: WT versus *Utx* KO, $64.7 \pm 4.6\%$ versus $57.4 \pm 1.4\%$ [$4.8 \pm 3.0 \times 10^3$ cells versus $5.0 \pm 2.4 \times 10^3$ cells] (Supplemental Fig. 3A and data not shown). The frequency and cell number of $CD4^{+}$ T_{RM}

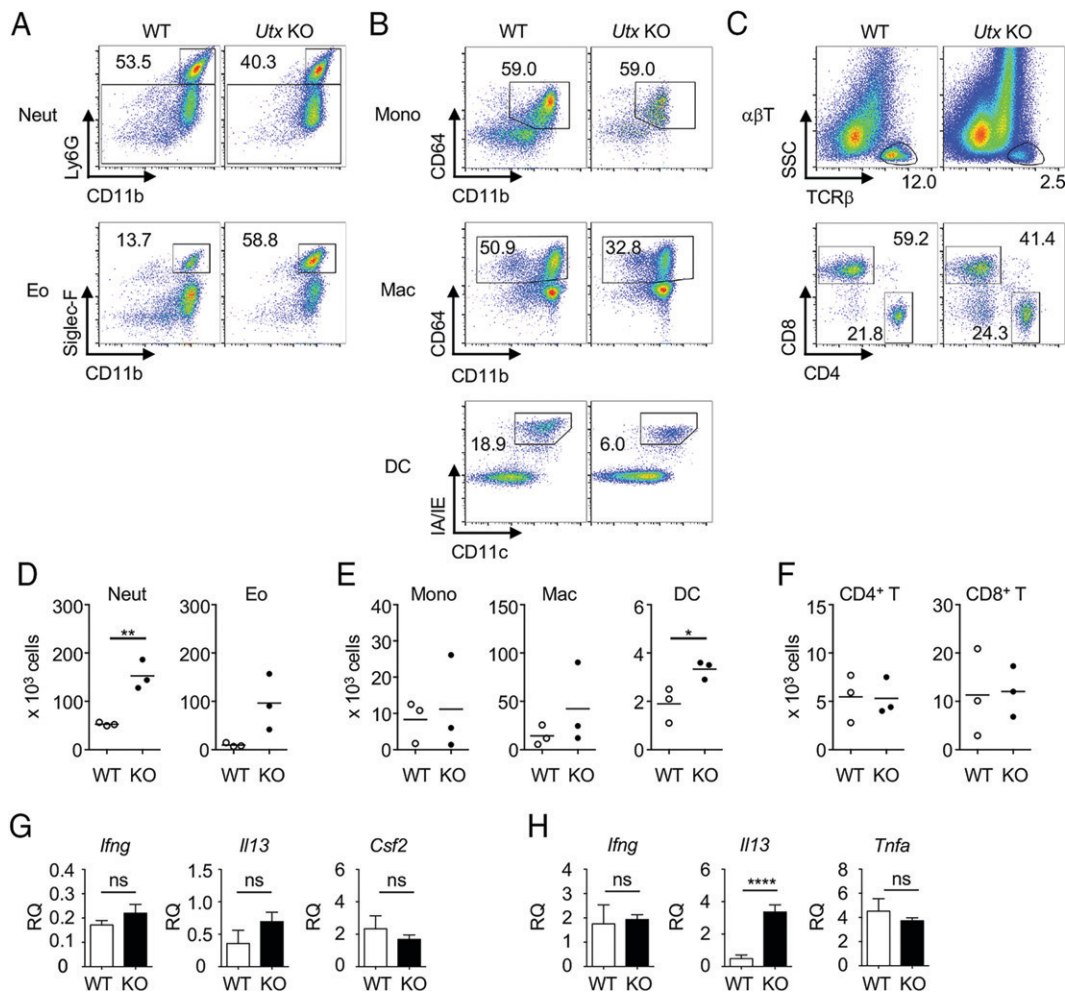


FIGURE 2. The cellular and functional analysis of immune cells that accumulated in the skin of *Utx* KO mice after repeated applications of a hapten DNFB. Plots of (A) granulocytes, including neutrophils (Neut; CD45⁺ lineage marker [Lin][−] TCR-β⁺ CD11b⁺ Ly6G⁺) and eosinophils (Eo; CD45⁺ Lin[−] TCR-β⁺ Ly6G[−] IA/IE[−] CD11b⁺ Siglec-F⁺), (B) monocytes (Mono; CD45⁺ Lin[−] Ly6G[−] IA/IE[−] SSC-A^{lo} CD11b⁺ CD64⁺), macrophages (Mac; CD45⁺ Lin[−] Ly6G[−] IA/IE^{+/hi} CD11b^{−/hi} CD64⁺), and DCs (CD45⁺ Lin[−] Ly6G[−] IA/IE^{hi} CD11b^{−/hi} CD11c⁺ CD64[−]) and (C) αβT cells (αβT, CD45⁺ TCR-β⁺ SSC-A^{lo}), CD4⁺ T cells (CD45⁺ TCR-β⁺ SSC-A^{lo} CD4⁺), and CD8⁺ T cells (CD45⁺ TCR-β⁺ SSC-A^{lo} CD8⁺). Numbers on plots indicate the frequencies of gated populations (A–C). Calculated cell numbers (per one piece of auricle) of neutrophils and eosinophils (D); calculated cell numbers (per one piece of auricle) of monocytes, macrophages, and DCs (E); and calculated cell numbers (per one piece of auricle) of CD4⁺ T cells and CD8⁺ T cells (F). The cell numbers in (D)–(F) were determined from three independent FACS analyses of pooled skin at 24 h after the third challenge with DNFB. WT (open circles) and *Utx* KO (filled circles). Each dot indicates one experiment. Three WT mice and two *Utx* KO mice were used in each experiment depicted in (A), (B), (D), and (E). Three WT mice and three *Utx* KO mice were used in each experiment shown in (B) and (E). Each bar indicates the mean. Profiles of the mRNA expression of the cytokine gene of (G) purified CD4⁺ T cells (CD45⁺ TCR-β⁺ SSC-A^{lo} CD4⁺) and (H) purified CD8⁺ T cells (CD45⁺ TCR-β⁺ SSC-A^{lo} CD8⁺) from pooled skin at 24 h after the third challenge with DNFB. RQ, relative quantity. Data are shown as levels relative to the reference gene, *18s* rRNA. WT (open bars) and *Utx* KO (filled bars). Error bars indicate technical variation in triplicate. Data represent the results of two independent experiments (G and H). Two-tailed unpaired *t* test; **p* < 0.05, ***p* < 0.01, *****p* < 0.001. Refer to Supplemental Fig. 2 related to (A) and (B) for the gating strategies. Supplemental Fig. 3 is related to (C); it shows the further analysis for memory phenotypes and CD25 expression.

cells (CD45⁺ Lin[−] TCR-β⁺ CD4⁺ CD69⁺ CD103^{+/−}) in the skin of *Utx* KO mice was slightly higher than that in the skin of WT mice, although the trend was not statistically significant: WT versus *Utx* KO, 19.3 ± 17.9% versus 31.4 ± 18.0% [1.1 ± 1.1 × 10³ cells versus 1.9 ± 1.8 × 10³ cells] (Supplemental Fig. 3B and data not shown). Similarly to CD4⁺ T cells, the numbers of CD8⁺ T_{EM} cells (CD45⁺ TCR-β⁺ CD8⁺ CD62L^{lo} CD44^{hi}) in the DNFB-treated skin of WT mice and *Utx* KO mice were statistically identical: WT versus *Utx* KO, 71.8 ± 13.0% versus 70.9 ± 14.8% [10.1 ± 7.6 × 10³ cells versus 5.3 ± 2.3 × 10³ cells] (Supplemental Fig. 3C and data not shown). In addition, the numbers of CD8⁺ T_{RM} cells (CD45⁺ TCR-β⁺ CD8⁺ CD69⁺ CD103⁺) in the skin of *Utx* KO mice were also statistically identical to those of WT mice (Supplemental Fig. 3D, WT versus *Utx* KO, 19.0 ± 4.5% versus 13.8 ± 0.5% [1.9 ± 1.4 × 10³ cells versus 1.7 ± 0.7 × 10³ cells]).

Although the numbers of CD4⁺ T cells and CD8⁺ T cells in the lesional skin were identical between WT and *Utx* KO mice, the numbers of CD25⁺ Foxp3[−] T_{conv} cells were significantly increased in the skin of *Utx* KO mice, whereas the numbers of Foxp3⁺ Treg cells were decreased in *Utx* KO mice (Supplemental Fig. 3E and 3F). The result suggests that a larger number of CD4⁺ T cells that accumulated in the lesional skin of *Utx* KO mice could become activated. The mRNA expression of inflammatory cytokine genes was analyzed at the same time point as Fig. 2A–2F to clarify the functionality of CD4⁺ T cells and CD8⁺ T cells in the skin (Fig. 2G and 2H). Total CD4⁺ T cells in the DNFB-treated skin of *Utx* KO mice expressed proinflammatory cytokine mRNAs, including *Ifng*, *Il13*, and *Csf2*, at levels almost identical to those of the cells of WT mice (Fig. 2G). Similarly, total CD8⁺ T cells in the skin of *Utx* KO mice expressed proinflammatory cytokines such as *Ifng* and *Tnfa* at

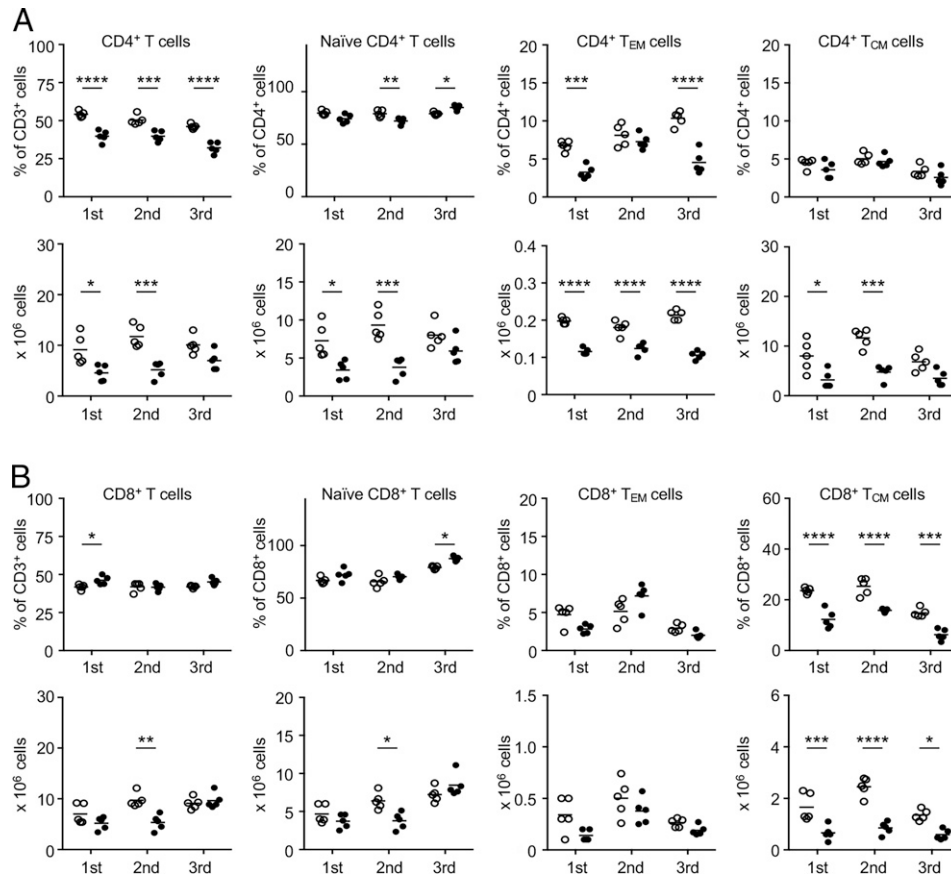


FIGURE 3. Existence of T cells at various stages in the draining LNs of challenged *Utx* KO mice after repeated applications of a hapten DNFB. The dynamics of frequency and cell numbers of (A) CD4⁺ T cells and (B) CD8⁺ T cells at various stages in the draining LNs at 24 h after the first, second, and third challenges with DNFB. WT (open circles) and *Utx* KO (filled circles). Each dot indicates one mouse (Ctrl, *n* = 5; *Utx* KO, *n* = 5). Two-way ANOVA followed by Sidak's post hoc test; **p* < 0.05, ***p* < 0.01, ****p* < 0.005, *****p* < 0.001. CM, CD62L^{hi} CD44^{hi} central memory; EM, CD62L^{lo} CD44^{hi} effector memory; Naive, CD62L^{hi} CD44^{lo}.

levels identical to those of the cells of WT mice. However, the *Il13* expression of *Utx* KO CD8⁺ T cells in the skin was significantly higher than that in WT cells (Fig. 2H). It is noteworthy that the relative quantities of *Ifng* and *Il13* in CD4⁺ T cells and CD8⁺ T cells were calculated using the same standardized relative quantity (Fig. 2G and 2H). The *Ifng* expression of WT CD8⁺ T cells was ~10 times higher than the expression of WT CD4⁺ T cells, whereas the *Il13* expression in WT CD8⁺ T cells and WT CD4⁺ T cells was identical at the time point that we tested. Taken together, repeated applications of DNFB to *Utx* KO mice caused the accumulation of larger numbers of neutrophils and DCs as well as possibly larger numbers of eosinophils and macrophages in the skin lesion than in WT mice. Furthermore, the activation of *Utx* KO CD4⁺ T cells and CD8⁺ T cell-derived proinflammatory cytokines such as IL-13 might be involved in the aggravation of skin symptoms.

Changes of CD4⁺ T cell phenotypes in the draining LNs after repeated application of DNFB

The sensitization of skin by having contact with haptens primes the naive T cells in the draining LNs into effector T cells through migratory DCs that present peptides from haptenated proteins (3, 4). To compare the kinetics of surface markers on T cells in the draining LNs along with Ag applications between WT mice and *Utx* KO mice, we analyzed naive, central memory (CM), and EM phenotypes on CD4⁺ or CD8⁺ T cells according to the expression of CD62L and CD44. As shown in Fig. 3A and 3B, the frequencies and cell numbers of total CD4⁺ and CD8⁺ T cells were both lower

in *Utx* KO mice at 24 h after the second challenge with DNFB. At this point, a significant difference in ear thickness was observed between WT and *Utx* KO mice (Fig. 1A). Furthermore, the numbers of CD62L^{hi} CD44^{lo} naive CD4⁺ T cells, CD62L^{lo} CD44^{hi} CD4⁺ T_{EM} cells, and CD62L^{hi} CD44^{hi} CD4⁺ T_{CM} cells were all decreased in *Utx* KO mice until 24 h after the second challenge, whereas the frequencies were dramatically decreased in CD4⁺ T_{EM} cells at 24 h after the third challenge (Fig. 3A). In contrast, significant differences in the frequencies and cell numbers in CD8⁺ T cells were only observed in the CM populations, with the exception of the slight difference in cell numbers of naive CD8⁺ T cells between WT and *Utx* KO mice (Fig. 3B). Because at least two challenges with DNFB caused swelling responses in the auricle skin that were more severe in *Utx* KO mice than in WT mice (Fig. 1A), the cell numbers of memory and/or naive phenotypes of CD4⁺ T cells and CD8⁺ T cells in the draining LNs might not be linked to the severity of the skin lesion.

To assess the capability of cytokine production in CD4⁺ T cells of skin-draining LNs after challenge with DNFB, we analyzed the proinflammatory phenotypes of CD4⁺ T cells and compared the cells between WT or *Utx* KO mice at 24 h after the first, second, and third challenges with DNFB. Among the cytokines that we tested, the *Ifng* and *Csf2* mRNA expression levels at 24 h after the second challenge with DNFB were higher, whereas the *Il17a*, *Il17f*, and *Il22* mRNA expression levels were lower, in *Utx* KO CD4⁺ T cells of the draining LNs than in WT CD4⁺ T cells (Fig. 4A). Consistent with the lower *Il17a* and *Il17f* mRNA expression in *Utx* KO

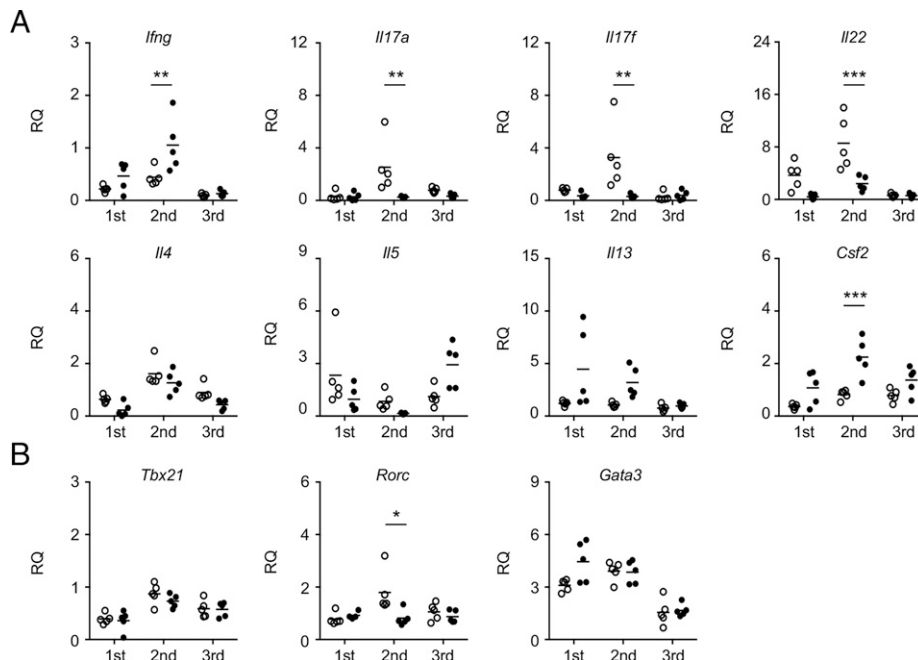


FIGURE 4. The expression of proinflammatory cytokines and master regulators in CD4⁺ T cells of the draining LNs of challenged *Utx* KO mice after repeated applications of a hapten DNFB. The dynamics of the expression levels of proinflammatory cytokines (**A**) and those of master regulators for Th1, Th17, and Th2 cells (**B**) in CD4⁺ T cells of the draining LNs at 24 h after the first, second, and third challenges with DNFB. RQ, relative quantity. Data are shown as levels relative to the reference gene, *18s* rRNA. WT (open circles) and *Utx* KO (filled circles). Each dot indicates one mouse (Ctrl, *n* = 5; *Utx* KO, *n* = 5). Two-way ANOVA followed by Sidak's post hoc test; **p* < 0.05, ***p* < 0.01, ****p* < 0.005.

CD4⁺ T cells, the master regulator for Th17 cell differentiation, *Rorc* (26), at 24 h after the second challenge with DNFB was lower in *Utx* KO CD4⁺ T cells than in WT CD4⁺ T cells. In CD4⁺ T cells, the expression levels of *Tbx21* coding T-bet, the master regulator for Th1 cells (27), and *Gata3*, the master regulator for Th2 cells (28), were identical in WT and *Utx* KO mice, regardless of the number of challenges with DNFB (Fig. 4B).

It is noteworthy that the expression of *Il13* was higher in *Utx* KO CD4⁺ T cells at 24 h after the first and second challenges with DNFB than that in WT CD4⁺ T cells. However, the difference was not statistically significant. The expression of *Il4* and *Il5* did not differ between *Utx* KO CD4⁺ T cells and WT CD4⁺ T cells, regardless of the number of challenges (Fig. 4A). The expression of *Gata3* dropped after the third challenge in CD4⁺ T cells, regardless of the presence of *Utx* (Fig. 4B). These findings suggested that *Utx* KO CD4⁺ T cells showed greater capacity to express *Ifng* and *Csf2* (and probably *Il13*) than did WT CD4⁺ T cells in the draining LNs after challenges with the hapten, but that *Utx* KO CD4⁺ T cells had a smaller capacity to express *Il17a*, *Il17f*, and *Il22* than did WT CD4⁺ T cells in the draining LNs after challenges with the hapten.

The roles of *Utx* in differentiating CD4⁺ T cells in vitro

To address the role of *Utx* in the functional differentiation of CD4⁺ T cells, we cultured splenic naive CD4⁺ T cells from either WT or *Utx* KO mice under various conditions. First, we analyzed the kinetic expression of *Kdm6a* and *Kdm6b* in naive CD4⁺ T cells after stimulation with anti-TCR-β plus anti-CD28 for up to 72 h (Fig. 5A). Interestingly, the kinetics of the expression of *Kdm6a* and *Kdm6b* in activated CD4⁺ T cells were different: The expression of *Kdm6a* was almost stable within the time frame that we analyzed, whereas the expression of *Kdm6b* was transiently increased at just after 2 h of stimulation and then gradually decreased (Fig. 5A). Second, the expression levels of *Kdm6a* in differentiating CD4⁺ T cells that were cultured for 2 d under neutral (ThN), Th1, Th2, Th17 or

iTreg conditions were all lower than those in naive CD4⁺ T cells. Similarly, the expression levels of *Kdm6b* in differentiating CD4⁺ T cells were all lower, regardless of the culture conditions (Fig. 5B).

To assess the effects of *Utx* deficiency in effector CD4⁺ T cell differentiation in vitro, we cultured naive CD4⁺ T cells from WT or *Utx* KO mice under various cytokine conditions. Activated *Utx* KO CD4⁺ T cells cultured with one ThN condition gave rise to a greater frequency of IFN-γ-producing cells and greater IFN-γ production than did activated WT CD4⁺ T cells. The *Utx* KO cells produced slightly a larger amount of IL-2 and a significantly larger amount of GM-CSF than did WT cells (Fig. 5C and Supplemental Fig. 4A). The increase in the production of IFN-γ and GM-CSF in *Utx* KO CD4⁺ T cells under one ThN condition was also supported by the status of H3K27me2 and H3K27me3 (Supplemental Fig. 4B). The basal level of H3K27me3 at the *Ifng* promoter region (*Ifng-p2*) in *Utx* KO unstimulated CD4⁺ T cells was lower than in WT unstimulated CD4⁺ T cells. After cultivation for 5 d, the level of H3K27me3 at the *Ifng-p2* in *Utx* KO cells showed no reduction, whereas the level in WT cells was decreased. The level of H3K27me2 at the *Csf2* promoter region (*Csf2-p*) of unstimulated *Utx* KO CD4⁺ T cells was almost the same as in unstimulated WT cells. However, the level of H3K27me2 at the *Csf2-p* gene locus of differentiated *Utx* KO cells was lower than that in differentiated WT cells (Supplemental Fig. 4B).

Similarly, *Utx* KO CD4⁺ T cells under one Th1 condition were capable of inducing slightly higher IL-2 production than were WT cells. To the contrary, the amount of IFN-γ in differentiated CD4⁺ T cells under one Th1 condition was slightly smaller than that in WT cells (Fig. 5D and Supplemental Fig. 4A). It is noteworthy that the amount of IFN-γ produced by *Utx* KO CD4⁺ T cells under one ThN condition (Fig. 5C) was almost identical to the amount produced under one Th1 condition (Fig. 5D). In addition, on the one hand, *Utx* KO CD4⁺ T cells that were cultured under one Th1 condition were capable of producing a significantly larger amount of GM-CSF than were WT CD4⁺ T cells (Fig. 5D). On the other

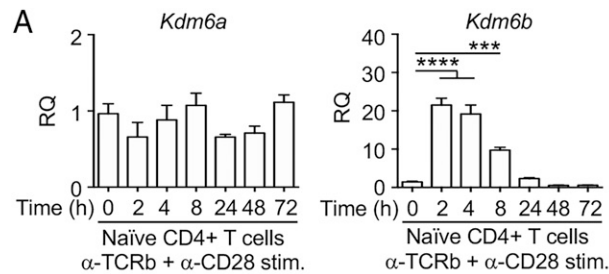
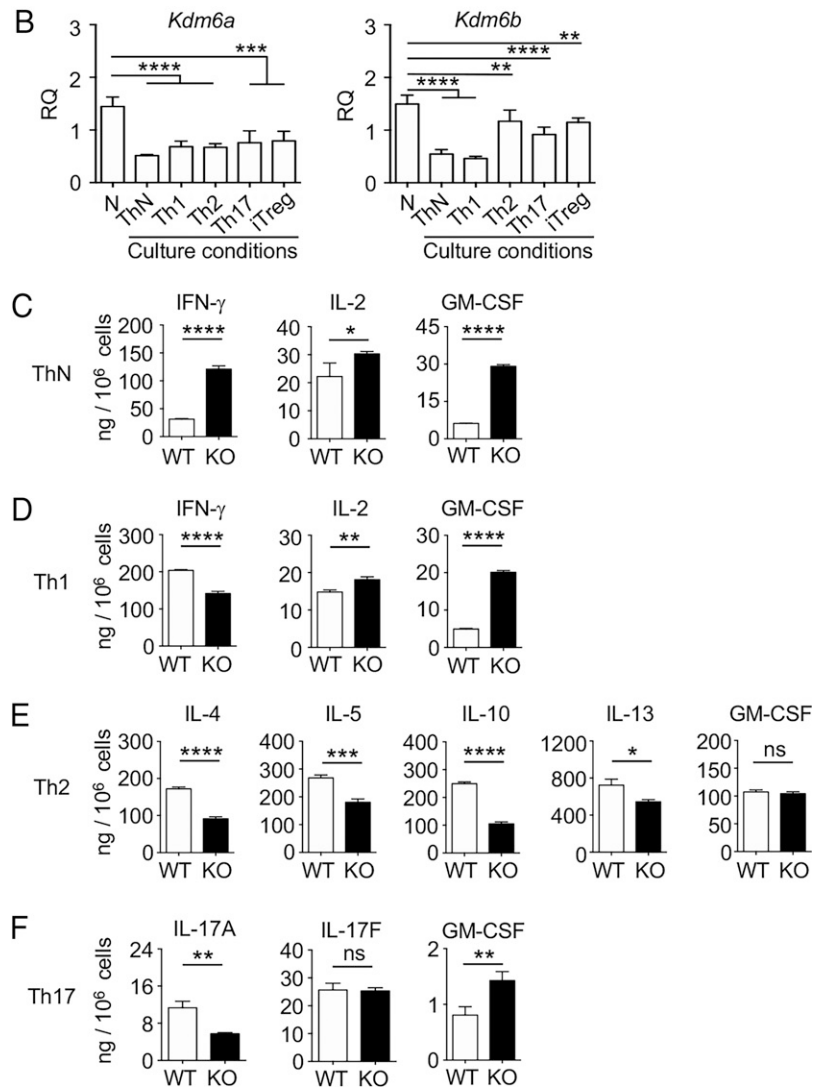


FIGURE 5. The roles of *Utx* in differentiating $CD4^+$ T cells in in vitro culture in the expression of proinflammatory cytokines and homing receptors in the skin. The expression levels of *Kdm6a* (left) and *Kdm6b* (right) in activating WT $CD4^+$ T cells under one ThN condition up to 72 h after TCR stimulation (**A**) and in differentiating $CD4^+$ T cells under the indicated culture conditions for 2 d (**B**). RQ, relative quantity. Data are shown as levels relative to the reference gene, *18s* rRNA. Error bars indicate technical variation. (A and B) One-way ANOVA, Tukey's post hoc test. $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$. (**C–F**) The amounts of cytokines produced in cultured $CD4^+$ T cells under (C) one ThN culture condition for 5 d, (D) one Th1 culture condition for 5 d, (E) one Th2 culture condition for 5 d, or (F) one Th17 culture condition for 3 d. The amounts of cytokines in the bar graphs in (C)–(F) indicate the amounts that were secreted by 1 million cells in the presence of anti-TCR- β mAb stimulation for 24 h. All the data represent the results of at least two independent experiments. Open bars, WT; filled bars, *Utx* KO. Error bars indicate technical variation. Two-tailed unpaired *t* test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$. Refer to Supplemental Fig. 4A [corresponding to (C)–(F)] for the frequency of cytokines producing $CD4^+$ T cells under indicated culture conditions, Supplemental Fig. 4B (corresponding to C) for the histone H3K27 methylation status at the cytokine and chemokine receptor gene loci, and Supplemental Fig. 4C [corresponding to (C)–(F)] for the expression of skin-tropic chemokine receptors.



hand, the capability to produce proinflammatory cytokines (e.g., IL-4, IL-5, and IL-13) and the regulatory cytokine IL-10 was decreased in *Utx* KO $CD4^+$ T cells cultured under one Th2 condition as compared with WT $CD4^+$ T cells, whereas the production of GM-CSF in WT and *Utx* KO $CD4^+$ T cells was identical (Fig. 5E and Supplemental Fig. 4A). The capability to produce IL-17A in *Utx* KO $CD4^+$ T cells cultured under one Th17 condition was also inhibited in comparison with WT $CD4^+$ T cells, whereas the IL-17F secretion was identical (Fig. 5F and Supplemental Fig. 4A). In addition, *Utx* KO $CD4^+$ T cells cultured under one Th17 condition were able to secrete larger amounts of GM-CSF than the WT $CD4^+$ T cells. However, the amount of GM-CSF produced by the *Utx* KO $CD4^+$ T cells under one Th17 condition was relatively much smaller than the amount of GM-CSF produced by the *Utx* KO cells under one Th2 condition (Fig. 5E and 5F).

The expression of chemokine receptors is required for the migration of $CD4^+$ T cells from the draining LNs to the skin (29). We therefore analyzed the expression of skin-tropic chemokine receptors, *Ccr4*, *Ccr8*, and *Ccr10*, on $CD4^+$ T cells cultured under various conditions in vitro. *Ccr4* was expressed at the highest level in differentiating Th2 and Th17 cells, whereas *Ccr8* and *Ccr10* were expressed in Th2 and ThN at the highest level in differentiating WT $CD4^+$ T cells, respectively. The loss of *Utx* slightly enhanced the *Ccr4*, *Ccr8*, and *Ccr10* expression in differentiating Th2 cells. In contrast, *Utx* KO Th1 cells showed lower *Ccr4* and *Ccr8* expression levels with the higher expression of *Ccr10*. *Utx* KO $CD4^+$ T cells cultured under one ThN condition also expressed a lower level of *Ccr10*. However, the expression levels of *Ccr4* and *Ccr8* in *Utx* KO ThN cells were identical (Supplemental Fig. 4C). These analyses of *Utx* KO $CD4^+$ T cells cultured under various conditions in vitro

suggested that activated *Utx* KO CD4⁺ T cells might be able to produce a large amount of IFN- γ and GM-CSF and that *Utx* KO effector CD4⁺ T cells could be prone to traffic to the skin.

T_{reg} cells in Utx KO mice with DNFB-derived ACD

Foxp3⁺ CD4⁺ T_{reg} cells play suppressive roles in hapten-specific effector responses (30). In order to address the effect of *Utx* deficiency in T_{reg} cells upon aggravation of DNFB-induced *Utx* KO ACD in mice and the role of *Utx* in the generation of T_{reg} cells in vitro, we first analyzed the frequencies and cell numbers of T_{reg} cells in our models of ACD. As shown in Fig. 6A and 6B, the frequency and cell numbers of Foxp3⁺ CD4⁺ T_{reg} cells in the skin of *Utx* KO mice at 24 h after the third challenge with DNFB were lower than those in WT mice. However, the changes of Foxp3⁺ CD4⁺ T_{reg} cells were not statistically significant: WT versus *Utx* KO, $7.1 \pm 1.2\%$ versus $2.1 \pm 0.4\%$ [$0.5 \pm 0.2 \times 10^3$ cells versus $0.2 \pm 0.1 \times 10^3$ cells]. Furthermore, the frequency and cell numbers of Foxp3⁺ CD4⁺ T_{conv} cells in the skin of *Utx* KO mice were higher. However, the changes of Foxp3⁺ CD4⁺ T_{conv} cells were not statistically significant: WT versus *Utx* KO, $40.8 \pm 5.1\%$ versus $61.9 \pm 3.7\%$ [$2.8 \pm 0.4 \times 10^3$ cells versus $5.2 \pm 1.8 \times 10^3$ cells]. The ratio of Foxp3⁺ CD4⁺ T_{reg} cells to Foxp3⁺ CD4⁺ T_{conv} cells in the skin was also lower in *Utx* KO mice than in WT mice: WT versus *Utx* KO, 0.18 ± 0.04 versus 0.04 ± 0.01 . Consistently, the expression of *Foxp3* in the CD4⁺ T cells in the skin of *Utx* KO mice was slightly lower than that in the cells in the skin of WT mice: WT versus *Utx* KO, 1.20 versus 0.97 (Fig. 6C). In addition to the phenotype in the skin, the frequencies and cell numbers of Foxp3⁺ CD4⁺ T_{reg} cells in the draining LNs of *Utx* KO mice and the ratio of T_{reg} cells to T_{conv} cells were all lower than those in WT mice, regardless of the number of DNFB challenges (Fig. 6D–6F). Furthermore, the expression of another T cell–mediated regulating factor, *Il10*, was also lower in CD4⁺ T cells of *Utx* KO mice than in those of WT mice at 24 h after the third challenge with DNFB, whereas the expression after the first and second challenges was identical (Fig. 6G). These data suggested that the regulatory functions of CD4⁺ T cells could become weaker without the expression of *Utx*.

In addition to the above analyses, we investigated the effect of the *Utx* deficiency in naive CD4⁺ T cells on the differentiation of T_{reg} cells in vitro. The dimethylation or trimethylation status of histone H3K27 at the *Foxp3* exon 1 region in *Utx* KO freshly isolated CD4⁺ and activated CD4⁺ T cells was almost comparable to that in WT cells (Supplemental Fig. 4B). However, the induction of Foxp3 in activated CD4⁺ T cells cultured under iTreg conditions was decreased in *Utx* KO CD4⁺ T cells as compared with that in WT CD4⁺ T cells (Fig. 6H). It is noteworthy that *Utx* KO Foxp3⁺ T_{reg} cells under one culture condition in the presence of TGF- β showed lower expression of CCR4 at both the mRNA (Fig. 6I) and protein (Fig. 6J) levels than did WT Foxp3⁺ T_{reg} cells. The efficiency of CD4⁺ T cell differentiation into T_{reg} cells and of CCR4 induction that is required for infiltration of the skin (31) was lower in the absence of *Utx*. All of the results suggested that lower numbers of Foxp3⁺ T_{reg} cells and the weaker expression of CCR4 in *Utx* KO CD4⁺ T cells could be linked to the aggravation of skin symptoms induced by the application of DNFB.

Discussion

Because CD4⁺ T cells are required to have an early inflammatory response for the induction of contact hypersensitivity (3), we focused on CD4⁺ T cells in the present study. We demonstrated that T cell–specific *Utx* KO mice showed severer symptoms of ACD than did WT mice, whereas *Jmjd3* KO mice showed

conditions of inflammation identical to those in WT mice. We therefore concluded that the following factors in *Utx* KO mice could potentially exacerbate the pathogenesis of ACD: (1) the massive accumulation of myeloid cells and the activation of Ag-specific T cells that express proinflammatory cytokines, (2) the decreased capability of CD4⁺ T cells to differentiate into Foxp3⁺ T_{reg} cells, and (3) the decreased expression of CCR4 (a skin-tropic chemokine receptor) in T_{reg} cells.

The analysis of the affected skin by flow cytometry showed a greater accumulation of myeloid cells, including neutrophils, DCs, eosinophils, and macrophages, in *Utx* KO mice than in WT mice. Furthermore, CD8⁺ T cells that were capable of producing larger amounts of proinflammatory cytokines (e.g., *Il13*) infiltrating the skin of *Utx* KO mice than were seen in the skin of WT mice after a third challenge with DNFB. Although the mRNA expression of cytokine genes in CD4⁺ T cells of the skin at one time point during the process of ACD development did not differ between *Utx* KO and WT mice to a statistically significant extent (Fig. 2G and 2H), the higher levels of cytokine expression, such as *Ifng* and *Csf2*, in *Utx* KO CD4⁺ T cells in the skin at a different time point, after a second challenge with DNFB, could be interpreted as follows. First, *Utx* KO CD4⁺ T cells expressed *Ifng* and *Csf2* mRNAs at higher levels than did WT CD4⁺ T cells during the development of ACD (Fig. 4A). Second, *Utx* KO CD4⁺ T cells cultured under one ThN condition gave rise to the expression of IFN- γ and GM-CSF at higher levels than did WT CD4⁺ T cells (Fig. 5C). The production of IL-13 could attract T cells via the induction of CCL22 in keratinocytes (32) and could downregulate the expression of structural molecules in the epidermis (33, 34). In addition, the IFN- γ expression in the skin could result in the accumulation of T cells and inflammatory DCs (35), the production of chemoattracting and activating factors for monocytes in keratinocytes (36), and the induction of the expression of transglutaminase, all processes that are involved in the cornification of keratinocytes (37). Furthermore, the presence of GM-CSF could become a mediator of tissue inflammation through DCs, macrophages, and granulocytes (38). Thus, the increase in the production of all proinflammatory cytokines in *Utx* KO T cells could be associated with the accumulation of the other immune cells in the skin, as we observed histologically in *Utx* KO mice. Further analysis of the kinetic expression of cytokines and comprehensive analysis of cytokine production in *Utx* KO T cells in the skin could further elucidate the mechanisms of skin symptoms of ACD.

As reported in one previous study (39), the expression of cell surface molecules, including CD44, may be affected by the deletion of *Utx* in CD4⁺ T cells. Throughout our analysis, we have observed the downregulated expression of CD44 in *Utx* KO T cells in the skin-draining LNs and spleen and a slightly downregulated expression level in the skin (Supplemental Fig. 3 and data not shown). Although CD44 may play a role in the development of ACD and the expression of CD44 in *Utx* KO T cells in the skin was relatively low (Fig. 2), we hypothesize that the lower expression of CD44 on *Utx* KO CD4⁺ T cells and CD8⁺ T cells could not affect their capability to express cytokines in the skin or the lesional skin.

Because Li et al. demonstrated that the generation of Foxp3⁺ T_{reg} cells was reduced in *Jmjd3*-deficient CD4⁺ T cells cultured under one iTreg condition in vitro (16), we examined whether the regulatory functions could become weakened in *Utx* KO mice. Strikingly, the numbers of T_{reg} cells in the skin of *Utx* KO mice were decreased, as were those in the draining LNs of *Utx* KO mice. The expression of the regulatory cytokine *Il10* was also decreased in both CD4⁺ T cells of *Utx* KO mice with ACD and *Utx* KO CD4⁺ T cells cultured under one Th2 condition in vitro. The decreased differentiation of *Utx* KO CD4⁺ T cells into Foxp3⁺ T_{reg}

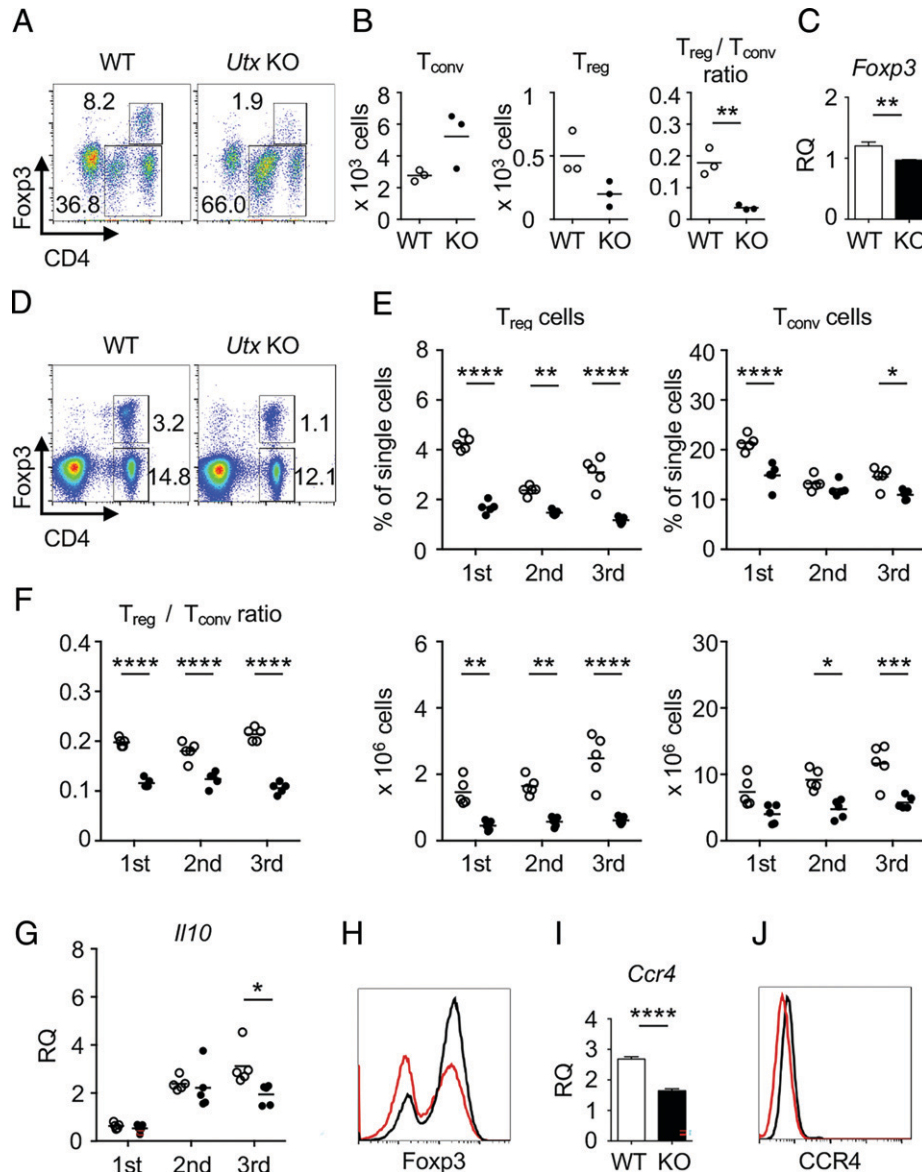


FIGURE 6. The existence of regulatory $CD4^+$ T cells in the skin and the draining LNs of *Utx* KO mice after repeated applications of DNFB. **(A)** Frequencies and **(B)** cell numbers of $CD45^+$ TCR- β^+ SSC-A ^{lo} $CD4^+$ Foxp3 $^+$ T_{reg} cells and $CD45^+$ TCR- β^+ SSC-A ^{lo} Foxp3 $^-$ $CD4^+$ T_{conv} cells in the pooled skin at 24 h after the third challenge with DNFB and the ratio of T_{reg} per T_{conv} cell numbers. WT (open circles) and *Utx* KO (filled circles). Each dot indicates one experiment. Five WT mice and five *Utx* KO mice were used in each experiment depicted in (A) and (B). Each bar indicates the mean. Two-tailed unpaired *t* test; $^{**}p < 0.01$. **(C)** the expression of *Foxp3* mRNA in $CD45^+$ TCR- β^+ SSC-A ^{lo} $CD4^+$ purified cells in the pooled skin from either WT or *Utx* KO mice at 24 h after the third challenge with DNFB. Open bars, WT; filled bars, *Utx* KO. Error bars indicate technical variation. Data represent the results of two independent experiments. Data are shown as levels relative to the reference gene, *18s* rRNA. Two-tailed unpaired *t* test; $^{**}p < 0.01$. **(D)** Frequencies of $CD3^+$ $CD4^+$ Foxp3 $^+$ T_{reg} cells and $CD3^+$ $CD4^+$ Foxp3 $^-$ T_{conv} cells in the draining LNs from either WT or *Utx* KO mice at 24 h after the third challenge with DNFB. The dynamics of **(E)** the frequency and cell number and **(F)** the ratio of $CD3^+$ $CD4^+$ Foxp3 $^+$ T_{reg} cells and $CD3^+$ $CD4^+$ Foxp3 $^-$ T_{conv} cells in the draining LNs from either WT or *Utx* KO mice at 24 h after the first, second, and third challenges with DNFB. **(G)** The dynamics of the *Il10* expression in the $CD4^+$ T cells of the draining LNs from either WT or *Utx* KO mice at 24 h after the first, second, and third challenges with DNFB. **(E–G)** WT (open circles) and *Utx* KO (filled circles). Each dot indicates one mouse (Ctrl, $n = 5$; *Utx* KO, $n = 5$). Two-way ANOVA followed by Sidak post hoc test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$. **(H)** The *Foxp3* expression, **(I)** the *Ccr4* mRNA expression, and **(J)** the CCR4 expression in differentiating T cells from either WT or *Utx* KO mice that were cultured under one iT_{reg} condition for 3 d. Data represent the results of two independent experiments. **(H and J)** WT (black lines) and *Utx* KO (red lines). **(I)** Open bars, WT; filled bars, *Utx* KO. Data are shown as levels relative to the reference gene, *18s* rRNA. Error bars indicate technical variation. Two-tailed unpaired *t* test, $^{****}p < 0.001$. RQ, relative quantity.

cells in in vitro culture under one iT_{reg} condition with the lower expression of CCR4 could be linked to the lower numbers of T_{reg} cells in both the skin and the draining LNs. All of the aspects that we have observed in *Utx* KO mice with ACD and in *Utx* KO $CD4^+$ T cells in vitro could be explained as the aggravation of the pathogenesis of ACD, partially due to the deterioration of the regulatory functions.

Based on our data on *Jmjd3* KO $CD4^+$ T cells in vitro (M. Omori-Miyake, unpublished observations), as well as the data described by Li et al. (16), the increase in IFN- γ production in in vitro cultures of *Utx* KO $CD4^+$ T cells under one ThN condition was not observed in *Jmjd3* KO $CD4^+$ T cells. Furthermore, *Utx* and *Jmjd3* deficiency had different influences on histone H3K27 methylation at the *Ifng* and *Csf2* promoter regions (Supplemental

Fig. 4B). Therefore, *Utx* and *Jmjd3* might have a distinct function for the differentiation of CD4⁺ T cells. Because the frequency of Ag-specific T cells in the draining LNs in response to an Ag could be quite low, we believe that the ThN conditions of culture, with only IL-2, would mimic the events in our experimental model of ACD. We therefore hypothesize that *Utx* could be involved in the Ag-driven expression of IFN- γ and GM-CSF in CD4⁺ T cells for the following reasons: (1) *Utx* KO CD4⁺ T cells expressed *Ifng* and *Csf2* mRNAs at higher levels than WT CD4⁺ T cells in response to the application of DNFB (Fig. 4); (2) the trimethylation status of the promoter region of the *Ifng* gene locus in *Utx* KO CD4⁺ T cells was originally lower than that in WT CD4⁺ T cells (Supplemental Fig. 4B); and (3) the TCR stimulation-driven downregulation of H3K27me2 status at the promoter region of the *Csf2* gene locus in *Utx* KO CD4⁺ T cells was greater in than that in WT CD4⁺ T cells (Supplemental Fig. 4B).

To envision our further studies, we also determined the cytokine-driven differentiation of *Utx* KO CD4⁺ T cells under Th1, Th2, Th17, or iTreg conditions in vitro (Fig. 5). The ablation of *Utx* in CD4⁺ T cells resulted in the decreased expression of each characteristic cytokine or transcription factor: IFN- γ for one Th1 condition; IL-4, IL-13, and IL-5 for one Th2 condition; IL-17A for one Th17 condition; and Foxp3 for one iTreg condition. In addition, the trends of IL-4, IL-5, and IL-13 production under one Th2 culture condition, the IL-17 production under one Th17 culture condition, and the Foxp3 expression under one iTreg culture condition in *Utx* KO CD4⁺ T cells and *Jmjd3* KO CD4⁺ T cells were all identical, as other groups have shown (Ref. 16 and M. Kuwahara, et al., manuscript posted on bioRxiv, DOI: 10.1101/184648). What these results suggest to us is that *Utx* and *Jmjd3* might have distinct function in a given condition but redundant functions in another condition for differentiation of CD4⁺ T cells. Although the molecular mechanisms of these changes in *Utx* KO CD4⁺ T cells remain to be investigated, the results have become a touchstone for our future studies.

In our present study, we assessed the effects of the loss of *Utx* and *Jmjd3* expression in T cells on the pathogenesis of ACD. The exacerbated symptoms of ACD in *Utx* KO mice could occur via the following potential mechanisms: the accumulation of T cells that secrete proinflammatory cytokines, the infiltration of proinflammatory myeloid cells that could be attracted and activated by the T cells, and the reduced number of T_{reg} cells in the skin. Further studies will be needed to elucidate each of these potential mechanisms and their interplay and thus to facilitate our further understanding of how and why the symptoms of ACD in *Utx* KO mice would be exacerbated.

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Disclosures

The authors have no financial conflicts of interest.

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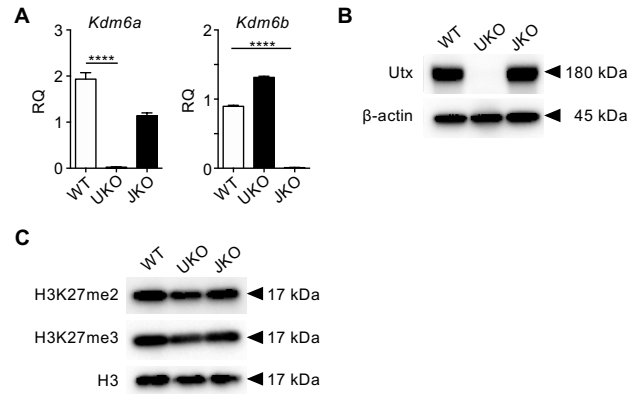


FIGURE S1. Expressions of *Utx* and *Jmjd3* and global methylation status of H3K27me2/3 in CD4⁺ T cells. (A) Messenger RNA expression of *Kdm6a* or *Kdm6b* was measured using primer pairs that were designed for the deleted region of exon 2 of *Kdm6a* gene for Utx detection and exon 17 of *Kdm6b* gene for Jmjd3 detection. Data were shown as relative levels to the reference gene, *18s* rRNA. RQ, relative quantity. Error bars indicate technical variation in triplicate. One-way ANOVA, Dunnett's post-hoc test, **** $p < 0.001$. (B) Immunoblot analysis of Utx and β -actin. Lysates of nuclear fraction for Utx and one of cytoplasmic fraction for β -actin. (C) Immunoblot analysis of histone H3, H3K27me2 and H3K27me3. Lysates from whole cells. Total RNAs (A) and cell lysates (B, C) were prepared from total CD4⁺ T cells on day 5 that were cultured with rmlL-2 in the presence of anti-TCR β and anti-CD28 for the first two days. WT, wild-type; UKO, *Utx* KO; JKO, *Jmjd3* KO.

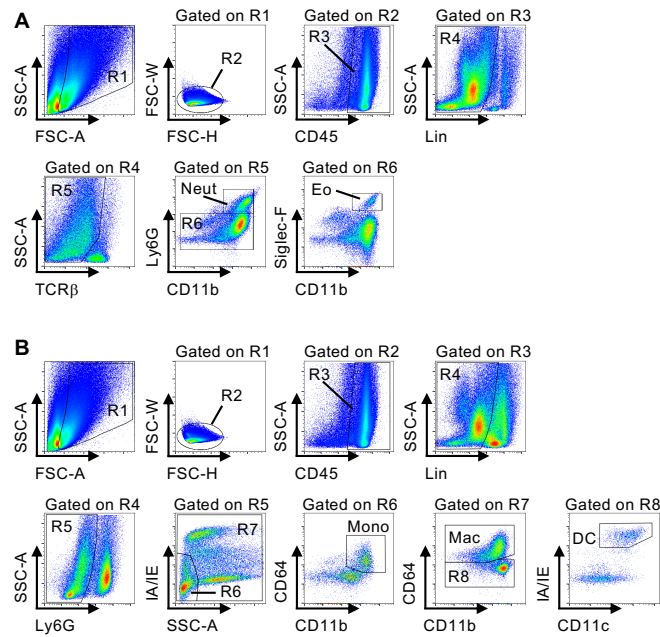


FIGURE S2. Gating strategies to identify innate cells in the mouse skin. **(A)** After live cells (FSC-A vs. SSC-A, R1) and single cells (FSC-H vs. FSC-W, R2) were included, leukocytes were gated as CD45⁺ cells (R3). Lineage marker⁻ (Lin⁻, R4) and TCRβ⁻ (R5) cells were gated and neutrophils were identified as Ly6G⁺ CD11b⁺. Eosinophils were identified as Siglec-F⁺ CD11b⁺ in Ly6G⁻ population (R6). TER119 and CD19 were both used as the lineage marker for **(A)**. **(B)** Leukocytes were gated as shown in **(A)**. After Lin⁻ (R4) and non-neutrophils (R5) were gated, monocytes were identified as CD64⁺ CD11b⁺ in IA/IE⁻ population (R6). Macrophages in R7 as CD64⁺ CD11b^{-high} and DCs in R8 as CD64⁻ CD11c⁺ IA/IE^{hi} were identified. TER119, CD19 and TCRβ were all used as the lineage marker for **(B)**. Neutrophils, Neut; eosinophils, Eo; monocytes, Mono; macrophages, Mac; dendritic cells, DC.

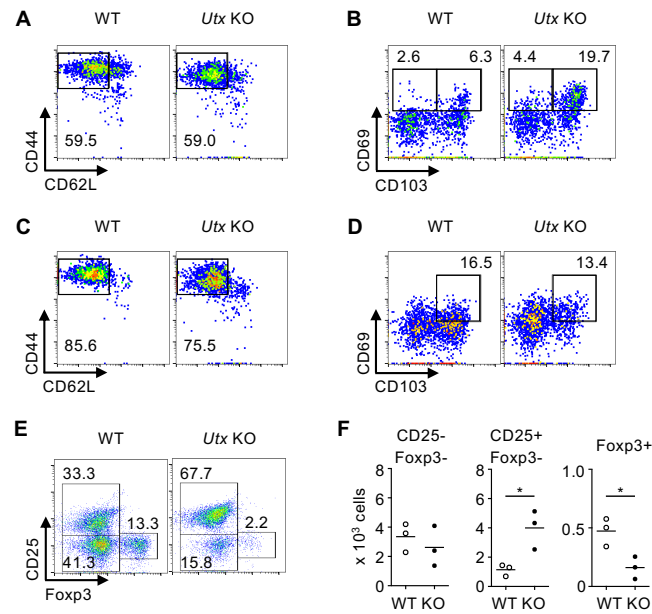


FIGURE S3. Flow-cytometric analysis of memory T cells in the mouse skin. Frequencies of CD62L^{lo} CD44^{hi} effector memory cells (**A**, **C**) and CD69⁺ resident memory cells (**B**, **D**) in CD4⁺ TCRβ⁺ cells (**A**, **B**) and CD8⁺ TCRβ⁺ cells (**C**, **D**) from pooled skin at 24 h after the 3rd challenge with DNFB. Number on plots indicate the frequencies of depicted gates. (**E**) Frequencies of CD25 in Foxp3⁻ or Foxp3⁺ CD4⁺ TCRβ⁺ cells from pooled skin at 24 h after the 3rd challenge with DNFB. (**F**) Cell numbers per auricle of CD25⁻ Foxp3⁻, CD25⁺ Foxp3⁻ and CD25^{int} Foxp3⁺ in CD4⁺ TCRβ⁺ were plotted from three independent experiment (n=3). Two-tailed unpaired t-test, * p<0.05.

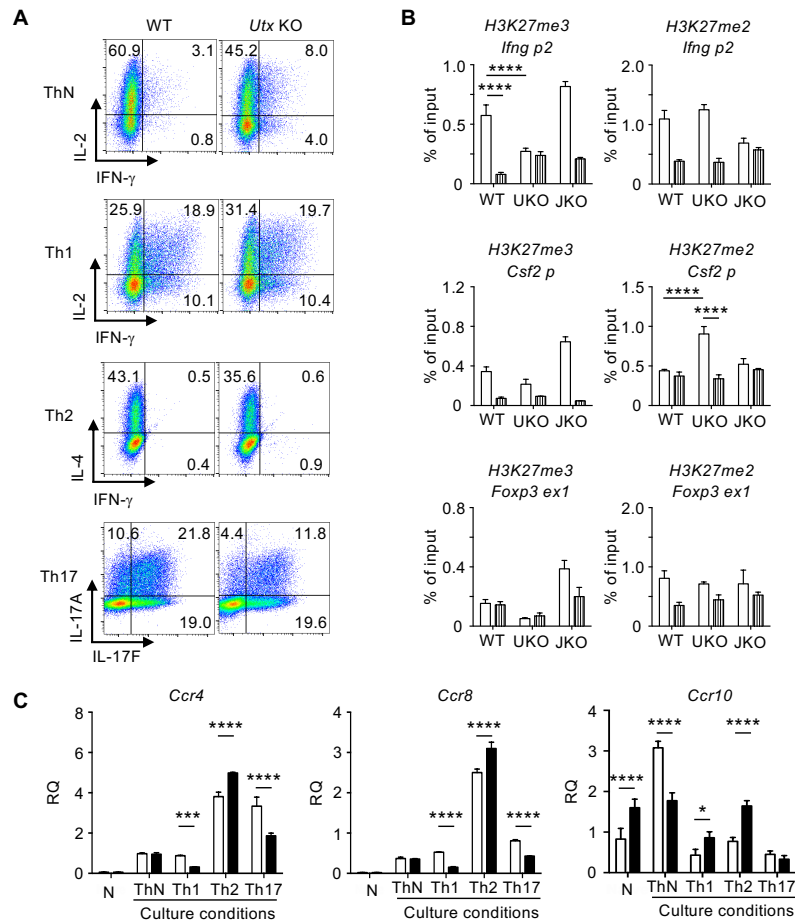


FIGURE S4. Locus specific methylation status of H3K27me2/3 in cultured CD4⁺ T cells. (A) The frequency of cytokines produced in cultured either WT or *Utx* KO CD4⁺ T cells under one ThN culture condition for five days, one Th1 culture condition for five days, one Th2 culture condition for five days, or one Th17 culture condition for three days. **(B)** ChIP-qPCR analysis on freshly isolated total CD4⁺ T cells from spleen and the cells that were cultured under the same condition as ThN in (A) with antibodies specific for di- or trimethylated H3K27. Data are shown as a percentage (%) of input DNA and indicate the distribution of di- or trimethylated H3K27 at indicated regions of genome. Opened bars, freshly isolated CD4⁺ T cells; Striped bars, cultured CD4⁺ T cells on day 5. WT, wild-type; UKO, *Utx* KO; JKO, *Jmjd3* KO. Two-way ANOVA, Tukey's post-hoc test, **** $p < 0.001$. **(C)** The expression levels of chemokine receptors relative to those homing to the skin in naïve (N) and differentiating CD4⁺ T cells that were cultured under the indicated culture conditions for two days. RQ, relative quantity. Data are shown as relative levels to the reference gene, *18s* rRNA. All the data represent the results of two independent experiments. Open bar, WT; closed bar, *Utx* KO. Error bars indicate technical variation. Two-way ANOVA, Sidak's post-hoc test, * $p < 0.05$, *** $p < 0.005$, **** $p < 0.001$.