International Immunology, Vol. 22, No. 4, pp. 329–340
doi:10.1093/intimm/dxq014
Advance Access publication 25 February 2010

Epidermal γδ T cells sense precancerous cellular dysregulation and initiate immune responses

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Transmitting editor: T. Saito

Received 5 November 2009, *accepted* 22 January 2010

Abstract

Hyperplasia associated with a loss of tissue homeostasis can induce DNA replication stress, leading to precancerous dysregulation. Epidermal γδ T cells reside in the primary barrier that protects against diverse environmental insults; however, the functions of these T cells in tissue surveillance are not completely understood. In mice with inducible *Notch1* inactivation in keratinocytes that causes epidermal hyperplasia, epidermal γδ T cells sensed stressed keratinocytes and migrated into the cutaneous draining lymph nodes. Simultaneous induction of β-galactosidase (β-Gal) as a putative antigen expressed in the process of precancerous dysregulation and *Notch1* ablation in the epidermis resulted in elevated β-Gal-specific IgG2a production. Epidermal γδ T cells were found to have the capacity to express chemokine (C-C motif) receptor 7 and migrate into the lymph nodes. Cutaneous draining lymph node cells in *Notch1*-inactivated mice expressed high levels of *IFN*-γ upon anti-CD3 plus anti-CD28 stimulation. Furthermore, induced expression of β-Gal in mice that lacked epidermal γδ T cells failed to induce anti-β-Gal IgG. These results suggest that epidermal γδ T cells play an essential role in the initiation process of epidermal antigen-specific humoral immune responses and demonstrate the importance of epidermal γδ T cells in sensing precancerous dysregulation and activating adaptive immunity.

Keywords: epidermal antigen-specific IgG, epidermal γδ, T cell, tissue surveillance

Introduction

The immune system is tightly regulated to react against invaded non-self-antigens and to avoid responses against self-antigens. T and B cells that react to self-antigens are either eliminated or suppressed during development. Precancerous cells that express a 'mutated-self'-antigen can also be targets of immune responses. The essential role of the adaptive immune response in eliminating these cells has been demonstrated in studies on immune-deficient or recombination activating gene 2 (RAG-2)-deficient mice that are more susceptible to carcinogens (1). Dormant tumors that grow in the absence of adaptive immunity are kept in check by the immune system (2). It is well established that the immune system detects pathogens using pattern recognition molecules that bind foreign microbes and induce innate immune responses that subsequently activate adaptive immune responses. Although T and B cells specific for tumor cells have been found in patients with tumors (3–5), it is not clear how innate immune responses initiate and activate antigen-specific immune responses. It is important to know how the immune system can sense precancerous changes in cells that activate the immune response to break established self-tolerance and prevent cancer development.

Anti-tumor immune responses are initiated by some innate immune responses (6). Abnormal cellular proliferation leads to genotoxic stress, which activates the DNA damage response, leading to the recruitment of DNA repair enzymes and elevated p53 protein levels (7). NKG2D ligands that are not expressed on the surface of normal cells are up-regulated during genotoxic stress (8). Expression of NKG2D ligands, including the Rae-1 family (α, β, γ, δ and ε), histocompatibility 60 (H60)

and murine UL-16-binding protein-like transcript 1 in mice and the MHC class I polypeptide-related sequence A and UL-16 binding protein families in humans (9, 10), can play a significant role in initiating the activation of NK cells and some other NKG2D-expressing cells. The initiation of immune responses may include some danger signals expressed by stressed cells. S100 proteins comprise a multi-gene family of low molecular weight calcium-binding proteins that can also be induced by stimuli that cause DNA damage such as ionizing radiation and UV irradiation (11). Many members of this gene family are encoded in the epidermal differentiation complex located on chromosome 1q21 in human and 3f2 in the mouse (12). S100A8 and S100A9 form homo- and heterodimers and are related to the hyperproliferative state of the epidermis. These proteins are often expressed in other tumors including adenocarcinomas and prostate and breast cancers. However, it is still unknown whether adaptive immune responses against tumor-specific antigens are also activated as a consequence of the process initiated during abnormal proliferation.

In the epidermis, there are two major populations of cells that have differentiated from hematopoietic precursor cells. Langerhans cells (LCs) are specialized dendritic cells (DCs) that capture and process antigens and then present them to T cells in the draining lymph nodes. These cells are thought to initiate adaptive immune responses against pathogenic organisms (13) but maintain self-tolerance by presenting self-antigens at steady state (14). LCs have been shown to regulate the immune response in some experimental systems since depletion of LCs augments contact hypersensitivity (15). Another cell type in the epidermis is the dendritic epidermal T cell that expresses γδ TCRs. These T cells express a canonical Vγ5/Vδ1 TCR and are generated by positive selection in the fetal thymus and migrate to the skin. The molecule, skint1, which is expressed both in the thymus and epidermis, has been identified recently to select these T cells (16). Unlike αβ T cells, γδ T cells recognize small molecular weight non-peptide, stress- or tumor-induced self-antigens (17). Other molecules recognized by murine γδ T cells include I-E^k, heat shock protein 65 and T10/22 (18). It has been demonstrated that the induction of an NKG2D ligand in the epidermis activates γδ T cells that inhibit carcinogenesis (19), indicating that one of the primary functions of epidermal γδ T cells is to eliminate stressed or transformed cells. γδ T cells have been shown to have additional roles in the peripheral blood, in which TCR stimulation results in the induction of the lymph node homing receptor, chemokine (C-C motif) receptor 7 (CCR7). These γδ T cells are found within germinal centers of B cell follicles, suggesting that γδ T cells play a role in humoral immunity (20). It has also been shown that innate lymphocytes, including γδ T cells, trigger DC maturation (21). However, it is not known whether γδ T cells in the epidermis also contribute to the induction of the adaptive immune response.

In order to address how and whether immune responses are initiated by sensing cellular dysregulation prior to tumor development, we used a tissue-specific model of inducible *Notch1* deletion in the epidermis, which results in hyperproliferation of epidermal keratinocytes and subsequent skin tumor development (22). Notch signaling in keratinocytes results in growth suppression by inducing $p21^{WAF1/Cip1}$, which is mediated by recombination signal binding protein for immunoglobulin kappa J region binding to the p21 promoter (23). A neo-self-antigen, a putative antigen expressed by gene mutations that may occur during cellular dysregulation, was expressed using the same inducible Cre recombination system. This inducible system is desirable because the process of precancerous changes and/or expression of a putative antigen can be observed at a given time specifically in the epidermis. In this study, we found that deleting *Notch1* in the epidermis resulted in cellular stress, leading to the up-regulation of p53 and *S100A8* expression without inflammatory cytokine induction. Epidermal γδ T cells were found to migrate to the cutaneous draining lymph nodes and help B cells produce IgG specific for an epidermal antigen. Epidermal γδ T cells appeared to have a unique ability to sense stressed keratinocytes and migrate to the draining lymph nodes where they activated B cells to contribute to the induction of adaptive immune response.

Methods

Animals

C57BL/6 mice were obtained from Sankyo Laboratories (Shizuoka, Japan). *keratin 14* (*K14*)-*CreER*^{T2} mice (24, 25) were crossed with mice carrying a floxed *Notch1* allele (26) and with mice carrying the *CAG-CAT-Z* transgene [(27), gift from Miyazaki]. Cre recombinase was activated by injecting 4-hydroxytamoxifen (OHT, Sigma, St Louis, MO, USA) dissolved in ethanol and then suspended in sunflower oil as described previously (28). Eight- to 12-week old mice were injected with 1 mg of OHT intra-peritoneally for 3 consecutive days. Ovalbumin (OVA)-specific OT-I (29) and OT-II (30) TCR transgenic mice were gifts from T. Tokuhisa and H. Kishimoto, respectively. *RAG2*$^{-/-}$ mice were housed in the Research Institute for Biological Sciences, Tokyo University of Science. *TCRα*$^{-/-}$ mice were a gift from T. Nakayama. All mice were backcrossed more than eight times onto the C57BL/6 background and housed under specific pathogen-free conditions. All experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of the Tokyo University of Science.

Preparation of epidermal cell suspensions

Epidermal sheets were prepared from ears that were split into dorsal and ventral sides and floated dermal side down for 1 h at 37°C in 0.2% trypsin (Gibco BRL, Grand Island, NY, USA) or shaved body wall skins treated with dispase II (Roche) and DNase (Sigma) as described previously (31) for western blot analysis. The epidermis was peeled off and the cells were suspended by mixing and pipetting the isolated epidermal sheets.

Antigen presentation assay

$CD4^+$ T cells were purified from spleen cells obtained from OVA-specific OT-II TCR transgenic mice using anti-CD4 magnetic-activated cell sorting (MACS) beads (Miltenyi Biotec, Auburn, CA, USA). Cells were carboxy fluorescein diacetate succinimidyl ester (CFSE)-labeled and total epidermal

cells were used to present OVA323–339 peptides. To analyze cross-presentation to $CD8^+$ OT-I T cells, $CD8^+$ T cells were purified from OVA-specific OT-I splenocytes with anti-CD8 MACS beads. Epidermal cells were incubated for 6 h with 1 mg ml^{-1} OVA and then washed and transferred into fresh medium. On day 3 of culture, LCs were enriched by HISTOPAQUE-1083 (Sigma) and purified with anti-CD11c magnetic beads (Miltenyi Biotec). Varying numbers of LCs were used to present OVA to OT-I $CD8^+$ T cells and ^{3}H-thymidine incorporation was measured.

Flow cytometry

Single-cell suspensions of epidermal cells or cutaneous draining lymph node cells were stained with primary antibodies followed by PE–Cy5–streptavidin or PE–Cy7–streptavidin. The following mAbs were used for multi-parameter flow cytometric analysis: FITC-, Allophycocyanin (APC)- or PE-conjugated anti-TCRγδ (e-Bioscience, San Diego. CA. USA), biotin-conjugated anti-CD3 (e-Bioscience), PE-conjugated anti-TCRβ (e-Bioscience), biotin-conjugated anti-NKG2D (e-Bioscience), FITC-conjugated anti-I-A (e-Bioscience), PE-conjugated anti-B220, PE-conjugated anti-CD11c (e-Bioscience), FITC-conjugated anti-mouse Vγ5 (BD Pharmingen, San Diego, CA, USA), APC-conjugated anti-CCR7 (BioLegend) and FITC- or PE–Cy5-conjugated anti-CD25 (e-Bioscience).

Histochemistry and immunoblotting

For hematoxylin and eosin (H&E) and β-galactosidase (β-Gal) histochemistry, 10-μm-thick frozen sections were cut with a cryostat (Leica) and then stained with X-gal (5 bromo-4-chloro-3-indolyl β-D-galactosidase) as described previously (32). Whole-cell protein extracts were prepared from epidermal cells and used for immunoblotting. Antibodies against Notch1 (sc-6041; Santa Cruz, Santa Cruz, CA, USA), mouse K5 (Covance, Princetone, NJ, USA) or α-tubulin (sc-5286, Santa Cruz) were used as probes. HRP-conjugated anti-goat or anti-mouse IgG was used as a second antibody.

Analysis of anti-β-Gal antibodies

Blood samples from the indicated mice were collected. ELISA plates were coated overnight with 50 ng β-Gal protein per well and then blocked with Assay Diluent (BD Biosciences). Serial dilutions of sera were adsorbed on the coated plates for 1 h. Bound antibody was detected with anti-mouse IgG (CHEMICON), anti-mouse IgG1 (BETHYL, Montgomery, TX, USA), anti-mouse IgG2a (BETHYL) or anti-mouse IgG2b (Southern Biotech, Birmingham, AL, USA).

Real-time reverse transcription–PCR

Total RNA was isolated from epidermal cell suspensions using RNeasy Fibrouse Tissue Midi Kits (Qiagen) or lymph node cells using Isogen (Wako, Osaka, Japan). Complementary DNA was prepared by reverse transcription using 1 μg RNA. Quantitative real-time PCR was performed with the Smart Cycler II System using SYBR Green Master Mix (TAKARA, Shiga, Japan). Values for each gene were normalized to the expression levels of hypoxanthine guanine phosphoribosyl transferase (HPRT) messenger RNA (mRNA). All samples were run in triplicate. The specific primers used for PCR were as follows—HPRT: 5′-TTGTTGTTGGATATGCCCTTGACTA-3′, 5′-AGGCAGATGGCCACAGGACTA-3′; IL-1α: 5′-AAGTTTGTCATGAATGATTCCCTC-3′, 5′-GTCTCACTACCTGTGATGAGT-3′; tumor necrosis factor (TNF-α): 5′-CATCTTCTCAAAATTCGAGTGACAA-3′, 5′-TGGGAGTAGACAAGGTACAACCC-3′; S100A8: 5′-GGAAATCACCATGCCCTCTA-3′, 5′-GCTGTCTTTGTGAGATGCCA-3′; S100A9: 5′-CCTGACACCCTGAGCAAGAAG-3′, 5′-CTTATCATTTCCCAGAACAA-3′; IL-6: 5′-CCACTTCACAAGTCGGAGGCTTA-3′, 5′-GCAAGTGCATCATCGTTGTTCATAC-3′, IL-4: 5′-TCTCGAATGTACCAGGAGCCATATC-3′, 5′-AGCACCTTGGAAGCCCTACAGA-3′; IFN-γ: 5′-ATGAACGCTACACACTGCATCTT-3′, 5′-GAATCAGCAGCGACTCCTTTTCC-3′; Vγ5-Jγ1: 5′-GCACTGGATCCAACTGAAAGAAG-3′, 5′-CAGAGGGAATTACTATGAGCTTAG-3′.

Contact hypersensitivity responses

To induce allergic contact dermatitis, mice were sensitized on day 0 by an epicutaneous application of 25 μl of 0.5% 2,4 dinitrofluorobenzene (DNFB, Sigma) in acetone:olive oil (4:1) to shaved abdominal skin. On day 5, baseline ear thickness was measured with an engineer's micrometer, after which the mice were challenged with 10 μl of 0.2% DNFB on both sides of one ear. Ears were measured 24 h after challenge and data were expressed as the ear swelling response above baseline (i.e. ear thickness 24 h after challenge minus baseline ear thickness).

Chemotaxis assay

Transmigration assays were performed using 24-well transwell chambers with a 5-μm-pore filter (Corning Costar, NY, USA). The epidermal cell suspension was placed on a filter at 1×10^6 per well and then incubated for 48 h at 37°C. The cells that transmigrated into the lower chambers containing either medium alone or chemokine (C-C motif) ligand 21 (CCL21) were recovered and analyzed by flow cytometry. Antibodies against CCL21 (R&D Systems) were used to block the migration.

Results

Inactivation of Notch1 in the epidermis results in stress-induced gene expression

Epidermal basal cells proliferate to maintain epidermal homeostasis. Ablation of *Notch1* in epidermal cells results in derepressed β-catenin signaling, which leads to hyperplasia (22). In order to determine if the tissue surveillance system can sense precancerous changes, ligand-dependent Cre recombination was used to mutate a specific gene at a given time (24, 25). *CreER^{T2}* driven by the *K14* promoter enables the Lox-flanked *Notch1* gene to be excised in the epidermis upon administration of OHT. *K14-CreER^{T2}* (*Cre*) mice and those carrying *Notch1$^{lox/lox}$* alleles (*Cre/Notch1$^{lox/lox}$*) were injected with OHT intraperitonealy for 3 consecutive days. On day 9, which was 1 week post-treatment, the epidermis was isolated from the abdominal and back skin, and Notch1 expression was analyzed. As shown in Fig. 1(A), Notch1 was detected as an intact 300 kDa protein and as a cleaved 120 kDa band in control mice, while both bands were virtually lost in *Cre/Notch1$^{lox/lox}$* mice. These *Notch1*-deleted epidermal

cells had a selective growth advantage and differed from normal proliferating keratinocytes because p53, which is induced by cellular stress, was up-regulated in *Notch1*-deleted (Notch1$^{-/-}$) epidermal cells (Fig. 1B). Chemically induced skin carcinogenesis is often correlated with sustained inflammation during tumor development. To determine if keratinocyte hyperproliferation caused by *Notch1* inactivation also up-regulated inflammatory mediators, the epidermis was isolated from both control and *Notch1*-inactivated mice 1 week after OHT administration and mRNA levels were measured by real-time PCR. The expression of *S100A8*, which is known to be up-regulated during skin carcinogenesis (33), was increased >10-fold. Another S100 protein, *S100A9*, was also slightly up-regulated in the *Notch1*-deleted epidermis (Fig. 1C). In addition, levels of the inflammatory cytokines *TNF-α*, *IL-1α* and *IL-6* were the same as in the control, suggesting that the *Notch1* inactivation-mediated skin disorder differed from normal proliferation and was distinct from inflammation caused by chemicals and pathogens.

Deletion of Notch1 enhances immune responses against a neo-self-antigen induced in the epidermis

During the course of tumor development when mutations of various genes are involved, a mutated gene product can

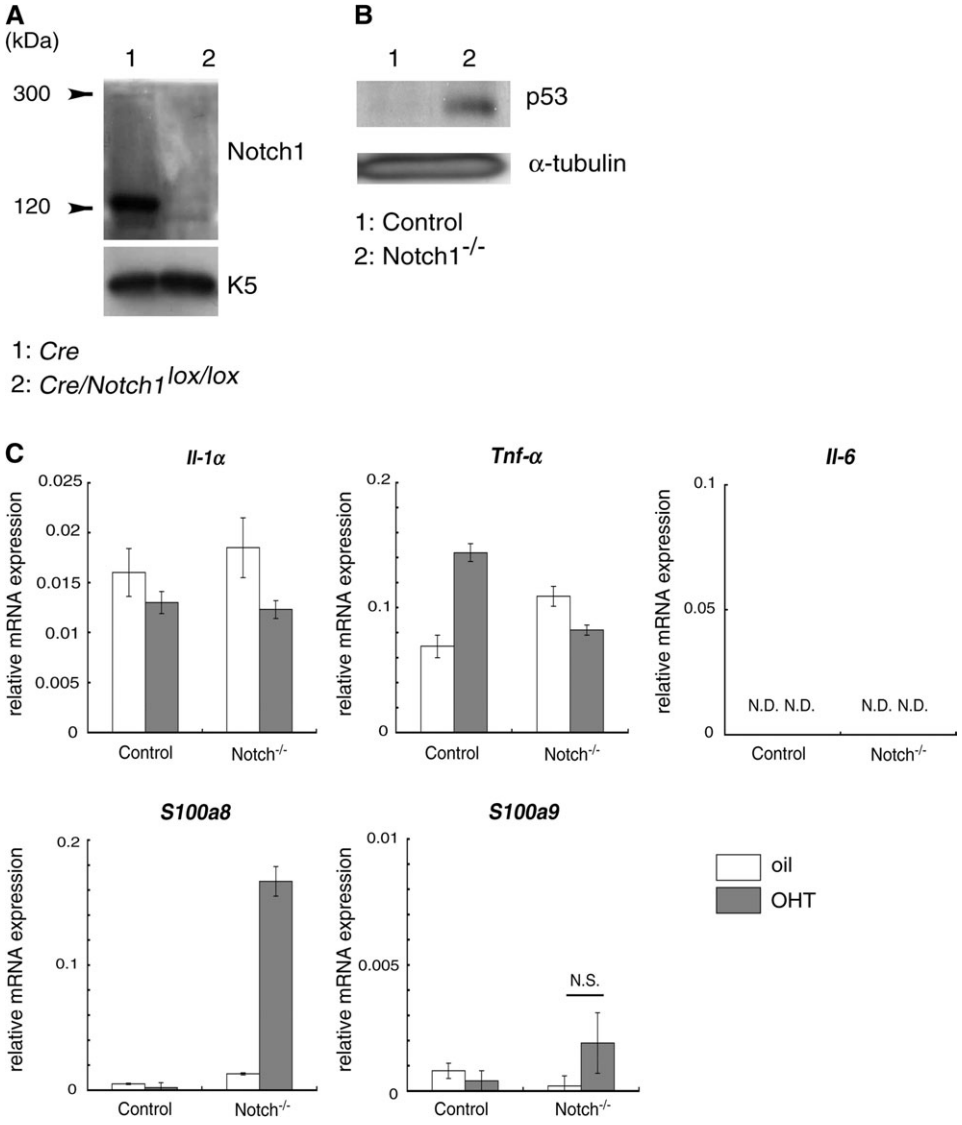


Fig. 1. *Notch1* inactivation in keratinocytes induces cellular stress. *K14-Cre-ER^{T2}* (lane 1: *Cre*) and *K14-Cre-ER^{T2}/Notch1$^{lox/lox}$* (lane 2: *Cre/Notch1$^{lox/lox}$*) were analyzed by immunoblotting and the same membrane was re-blotted with anti-keratin 5. Arrows indicate the 300 and 120 kDa (cleaved) bands. (B) Induction of p53 was analyzed in epidermal cells isolated from control (lane 1) and *Notch1*-inactivated (lane 2: Notch1$^{-/-}$) mice 1 week after OHT injection. Total cell lysates extracted from several Notch1$^{-/-}$ mice had similar expression levels. A representative blot is shown. (C) Epidermal cell suspensions were prepared from control and *K14-Cre-ER^{T2}/Notch1$^{lox/lox}$* (Notch1$^{-/-}$) mice 1 week after treatment. Total RNA was extracted from epidermal cells. Real-time reverse transcription–PCR was performed with primers for the indicated genes and the results were normalized to HPRT mRNA levels. Relative expression levels of each gene in oil-injected (open columns) and OHT-injected (closed columns) mice are shown. Data are representative of three independent experiments. Error bars indicate standard deviation. ND = not detected. NS = not significant.

become a neo-self-antigen. Since we were interested in determining whether such an antigen expressed prior to tumor development could be a target of immune responses, the same inducible Cre recombination system was used to induce the expression of a putative antigen in the epidermis. We first tested whether ectopic induction of a neo-self-antigen in the epidermis could lead to antigen-specific immune responses. Cre-mediated recombination using *K14-CreER^{T2}* was performed for the *CAG-CAT-Z* transgene (27), where the *CAT* gene within the *loxP* sites was excised upon Cre recombination, resulting in β-Gal expression in the epidermis 2 days after OHT administration (Fig. 2A). Although the epidermis with induced β-Gal expression became slightly thicker in 2 weeks, no mononuclear cells infiltrated into the epidermis for up to 17 weeks (Fig. 2B). LCs in the epidermis are known to capture antigens and migrate into the draining lymph nodes where they present these antigens to T cells. However, there was no sign of β-Gal-specific T cell activation in the draining lymph nodes as lymph node cells obtained 2 weeks after OHT administration did not express cytokines (*IL-2*, *IL-4* and *IFN-γ*) upon β-Gal stimulation (data not shown). Interestingly, although β-Gal-specific T cell responses were not detected, β-Gal-specific IgG production was induced within 1 week after OHT administration and increased until 3 weeks (Fig. 2C). These results indicated that a newly synthesized antigen in keratinocytes induced antigen-specific immune responses, but the responses were transient and mice induced to express β-Gal (β-Gal$^+$) did not develop any dermatitis.

We next constructed a model in which expression of a neo-self-antigen and aberrant proliferation were induced as a consequence of gene mutations during tumor development. Deletion of *Notch1* and expression of *β-Gal* occurred simultaneously upon OHT administration in *K14-CreER^{T2}/CAG-CAT-Z/Notch1$^{lox/lox}$* mice. In mice induced to express *β-Gal* and delete *Notch1*, external changes such as hair loss on the ventral skin were frequently observed 2 weeks after OHT administration. Similar but minor changes were sometimes observed in *Notch1*-deleted (Notch1$^{-/-}$) mice; however, mice with *Notch1* deletion plus *β-Gal* expression (β-Gal$^+$/Notch1$^{-/-}$) had a severe phenotype (Fig. 3A). H&E staining of skin sections from β-Gal$^+$/Notch1$^{-/-}$ mice revealed epidermal hyperplasia containing epidermal cell layers with flat shapes, indicating that there were dramatic responses in the β-Gal$^+$/Notch1$^{-/-}$ epidermis (Fig. 3B). However, a single mutation, either inactivating *Notch1* or expressing *β-Gal*, was not sufficient to induce these responses. In addition, the serum levels of anti-β-Gal IgG in β-Gal$^+$/Notch1$^{-/-}$ mice were much higher than those in β-Gal$^+$ mice (Fig. 3C). Among the anti-β-Gal IgG isotypes, levels of IgG2a were significantly elevated in β-Gal$^+$/Notch1$^{-/-}$ mice, while those of IgG1, which was a dominant isotype after β-Gal immunization and β-Gal induction in the epidermis, were even lower in *Notch1*-deleted mice (Fig. 3D). Cutaneous draining lymph node cells obtained from β-Gal$^+$ and β-Gal$^+$/Notch1$^{-/-}$ mice 2 weeks after OHT administration were stimulated with either β-Gal or anti-CD3 plus anti-CD28 antibodies. *IFN-γ* expression was elevated in β-Gal$^+$/Notch1$^{-/-}$ mice stimulated with anti-CD3 plus anti-CD28 but not with β-Gal (Fig. 3E). IL-4 expression was poor in both β-Gal$^+$ and β-Gal$^+$/Notch1$^{-/-}$ mice. These results suggested that the high IgG2a levels in β-Gal$^+$/Notch1$^{-/-}$ mice were due to the elevated levels of IFN-γ produced by T cells but possibly not by β-Gal-specific CD4$^+$ T cells.

Enhanced immune responses in the Notch1-inactivated epidermis are LC independent

LCs play an important role in presenting epidermal antigens in the draining lymph nodes. To determine whether the high IgG production was due to the functional activation of LCs, the ability of LCs to present antigens was analyzed. OVA was pulsed on epidermal cells isolated from both Notch1$^{-/-}$ and control mice and the proliferation of CFSE-labeled CD4$^+$ T cells purified from OVA-specific TCR transgenic mice (OT-II) was measured. As shown in Fig. 4(A), OVA-specific T cells responded to both control and Notch1$^{-/-}$ APC similarly. LCs are also known to cross-present antigens derived from the skin (13). Therefore, epidermal cells were pulsed with OVA protein, and LCs purified from epidermal cell cultures were tested for their ability to activate CD8$^+$ T cells obtained from OVA-specific TCR transgenic mice (OT-I). Again, we did not find any differences in the ability of control and Notch1$^{-/-}$ to cross-present antigens (Fig. 4B). Contact

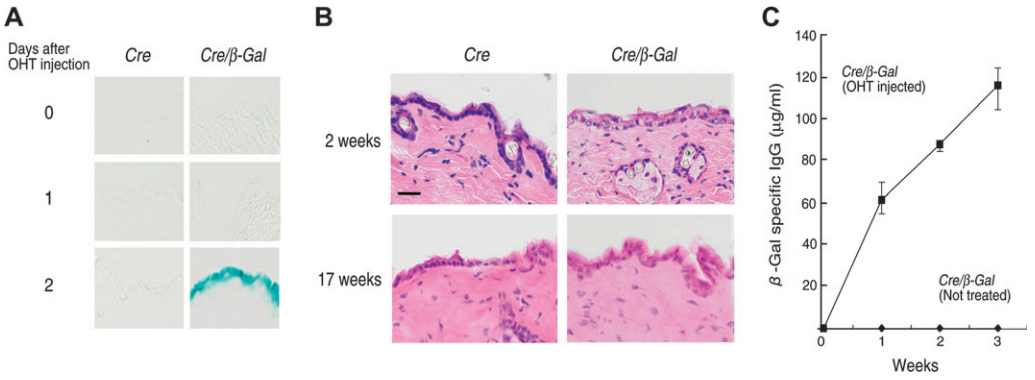


Fig. 2. Induced expression of β-Gal in keratinocytes leads to anti-β-Gal IgG production. (A) X-Gal staining of skin sections isolated from *K14-Cre-ER^{T2}* (*Cre*) and *K14-Cre-ER^{T2}/CAG-CAT-Z* (*Cre/β-Gal*) mice administered with OHT. (B) Sections of *Cre* and *Cre/β-Gal* back skin obtained 2 and 17 weeks after OHT administration were stained with H&E. Scale bar, 100 μm. (C) Serum IgG levels specific for β-Gal in *Cre/β-Gal* mice with or without OHT administration were analyzed. Data represent the means ± SDs and are representative of five independent experiments.

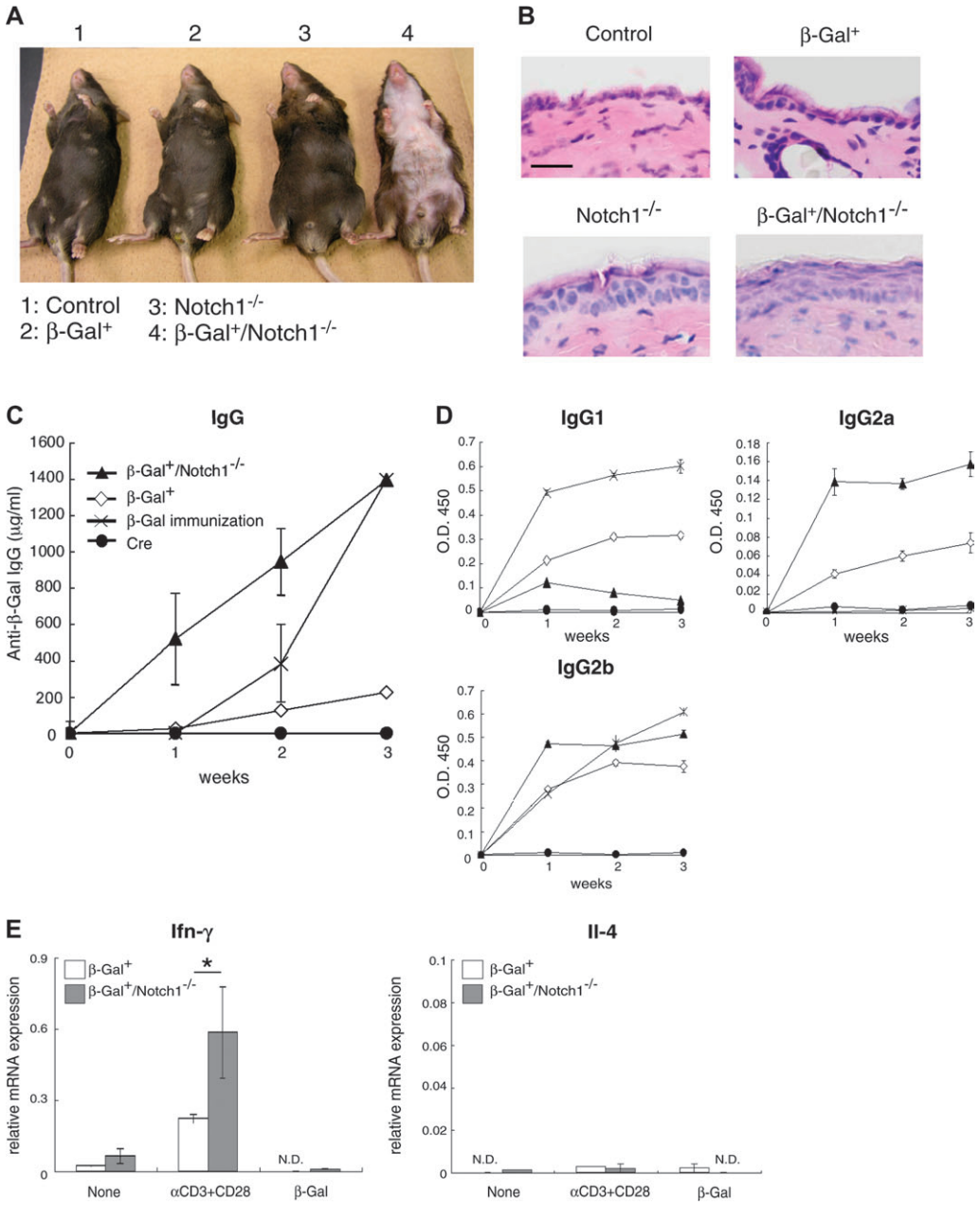


Fig. 3. Simultaneous induction of β-Gal expression and *Notch1* ablation leads to skin disorder and augmentation of anti-β-Gal IgG. (A) The indicated set of mice was analyzed 2 weeks after OHT administration. One representative analysis out of three independent experiments is shown. (B) Sections of the ventral part of the skin obtained from individual mice are shown (H&E stain). Scale bar, 100 μm. (C) Cre (closed circles), β-Gal$^+$ (open diamonds), β-Gal$^+$/Notch1$^{-/-}$ (closed triangles) mice injected with OHT and wild-type control mice immunized with β-Gal (β-Gal immunization, a line with cross symbols) were analyzed. Serum levels of anti-β-Gal IgG were measured by ELISA. One representative analysis out of three independent experiments is shown. Mean values ± SDs of triplicate assays are shown. (D) Anti-β-Gal IgG isotypes were further determined from the same serum samples analyzed in (C). Mean values of triplicate analyses are shown (mean ± SD). (E) Inguinal and axillary lymph node cells obtained from β-Gal$^+$ and β-Gal$^+$/Notch1$^{-/-}$ mice were cultured either with anti-CD3 plus anti-CD28 or β-Gal. The expression of *IFN-γ* or *IL-4* was analyzed by real-time reverse transcription–PCR (mean ± SD). Data from one representative assay out of three independent experiments are shown. ND = not detected. *, $P < 0.05$ (Student's *t*-test).

hypersensitivity is a classical skin response in which LCs are considered to play a role. *Notch1*-deleted and control mice were sensitized with DNFB and swelling associated with the response after DNFB challenge to the ears was measured. Although the ears of *Notch1*-deleted mice were slightly thicker because of epidermal hyperplasia, DNFB-specific changes in *Notch1*-deleted and control mice were in the same range (Fig. 4C). In addition, migration of LCs in Notch1$^{-/-}$ mice was normal (data not shown). These results suggested that LCs were not functionally altered by *Notch1* inactivation to augment anti-β-Gal T cell responses and induce antibody production in β-Gal$^+$/Notch1$^{-/-}$ mice.

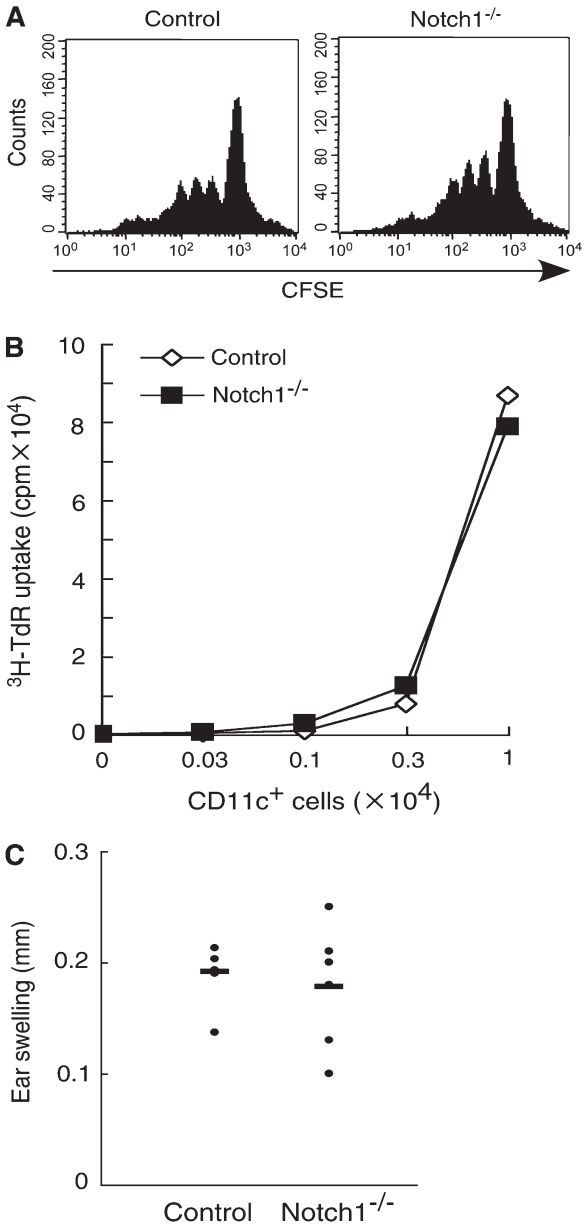


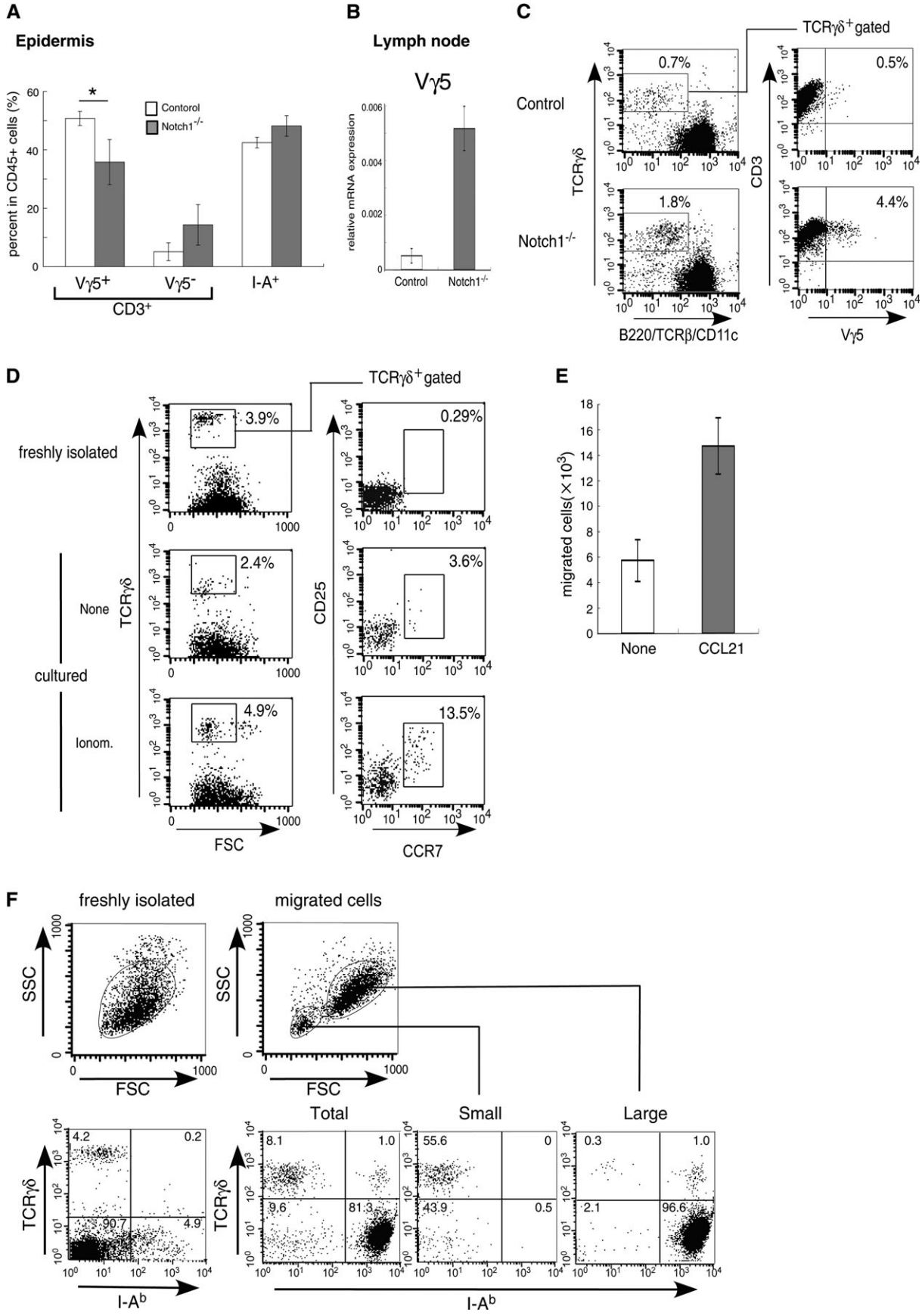
Fig. 4. LCs are not functionally altered in *Notch1*-inactivated mice. (A) Epidermal cell suspensions were prepared from wild-type (control) and *Notch1*-inactivated (Notch1$^{-/-}$) mice 2 weeks after OHT administration. OVA-specific CD4$^+$ T cells isolated from OT-II mice were labeled with CFSE and cultured with epidermal cells in the presence of OVA peptide. Data are representative of three independent experiments. (B) CD11c-positive cells were purified from epidermal cell suspensions that had been pulsed with OVA for 6 h and cultured for 2 days. OVA-specific CD8$^+$ T cells isolated from OT-I mice were incubated with increasing numbers of CD11c$^+$ cells, and ^{3}H-thymidine incorporation was measured after 2 days. Data are representative of three independent experiments. (C) Mice were treated with OHT 2 weeks before DNFB sensitization. Contact hypersensitivity responses were measured 24 h after the challenge. Control (n = 6) and induced Notch1$^{-/-}$ (n = 6) mice are shown (mean values are indicated by bars).

Activation of epidermal γδ T cells results in the induction of a lymph node homing program

We next examined whether epidermal γδ T cells are involved in β-Gal-specific IgG production. A previous report showed that γδ T cells can interact with B cells and induce Ig class switching (34). In this study, the percentages of epidermal γδ T cells expressing Vγ5 were decreased in the *Notch1*-inactivated epidermis, which was in contrast to the infiltration of Vγ5$^-$ T cells (Fig. 5A). The ratio of Vγ5$^+$ cells in the draining lymph nodes was analyzed by measuring mRNA levels of rearranged Vγ5-Jγ1. It was found that Vγ5 expression in the draining lymph nodes was increased significantly in Notch1$^{-/-}$ mice (Fig. 5B). Increased numbers of Vγ5$^+$ cells in TCRγδ$^+$, TCRβ$^-$, B220$^-$ and CD11c$^-$ cells were also found in the draining lymph nodes in Notch1$^{-/-}$ mice (Fig. 5C). These results suggested that epidermal γδ T cells expressing Vγ5 were activated and migrated into the draining lymph nodes.

Since epidermal γδ T cells recognize unknown antigens expressed in stressed keratinocytes (17), epidermal cell suspensions were cultured *in vitro* in the presence or absence of an artificial stimulus, ionomycin, to determine whether γδ T cells can express the lymph node homing receptor, CCR7. TCRγδ expression levels in cultured cells were lower than those in freshly isolated cells, which might be due to TCRγδ engagement. Cultured epidermal cells contained γδ T cells expressing both CCR7 and CD25, and ionomycin stimulation increased the number of those cells (Fig. 5D). To determine whether CCR7 expressed in epidermal γδ T cells is functional, we examined the chemotaxis of epidermal cells in response to the chemokine CCL21. An epidermal cell suspension was placed in a transwell chamber and cells that migrated out of the chamber were analyzed by FACS. The number of cells recovered in the bottom chamber containing CCL21 was much higher (Fig. 5E) and those cells responding to CCL21 contained both I-A$^+$ LCs and γδ T cells. TCRγδ$^+$ cells were in a fraction of small cells, expressing lower levels of TCRγδ than freshly isolated cells. Similar results were obtained by stimulating γδ T cells with anti-TCRγδ antibodies. Vγ5$^+$ cells were found in the population of small cells migrated in response to CCL21 (Supplementary Figure 1A, available at *International Immunology* Online), and the migration of Vγ5$^+$ cells was blocked by adding anti-CCL21 antibodies (Supplementary Figure 1B, available at *International Immunology* Online). I-A$^+$ cells, however, expressed higher levels of I-A molecules and were distributed mostly in a large cell population (Fig. 5F). These results suggested that γδ T cells became mobile in response to factors expressed by stressed keratinocytes and were targeted to draining lymph nodes expressing CCL21.

The molecules expressed by stressed keratinocytes that activate γδ T cells have not been identified. It was considered that abnormal keratinocyte proliferation might have induced DNA damage responses because the DNA damage checkpoint gene *p53* was already expressed in this early precancerous stage (Fig. 1B). In these cells, however, NKG2D ligands that are induced as a result of DNA damage in keratinocytes (19) were not detected in the *Notch1*-inactivated epidermis (data not shown). Therefore, γδ T cell activation in the *Notch1*-inactivated epidermis did not seem to be mediated by the NKG2D receptor.


A
Epidermis
percent in CD45+ cells (%)
Contorol
Notch1-/-
Vγ5+
Vγ5-
I-A+
CD3+
B
Lymph node
Vγ5
relative mRNA expression
Control
Notch1-/-
C
TCRγδ+gated
Control
Notch1-/-
0.7%
0.5%
1.8%
4.4%
TCRγδ
CD3
B220/TCRβ/CD11c
Vγ5
D
TCRγδ+gated
freshly isolated
cultured
None
Ionom.
3.9%
2.4%
4.9%
0.29%
3.6%
13.5%
TCRγδ
CD25
FSC
CCR7
E
migrated cells(×10³)
None
CCL21
F
freshly isolated
migrated cells
SSC
FSC
Total
Small
Large
4.2
0.2
90.7
4.9
8.1
1.0
9.6
81.3
55.6
0
43.9
0.5
0.3
1.0
2.1
96.6
TCRγδ
I-Ab

Activation of B cells to produce IgG specific for an epidermal antigen

Epidermal γδ T cells migrated in the draining lymph nodes might activate B cells directly or in collaboration with other antigen presenting cells. In order to determine whether epidermal γδ T cells are required for β-Gal-specific IgG production, we reconstituted *Cre/β-Gal/RAG2*$^{-/-}$ mice with normal spleen cells. These mice lack epidermal γδ T cells because they are generated during early ontogeny (Fig. 6A); however, peripheral lymphocytes are restored by the transferred splenocytes (Fig. 6B). OHT administration in the reconstituted mice did not induce anti-β-Gal IgG production, although *Cre/β-Gal* mice injected with OHT produced anti-β-Gal IgG (Fig. 6C). Since control *RAG2*$^{-/-}$ mice reconstituted with the same number of B6 splenocytes produced reasonable levels of anti-β-Gal IgG after immunization, the defect in IgG production in *Cre/β-Gal/RAG2*$^{-/-}$ mice after OHT administration could be due to the absence of epidermal γδ T cells. In order to determine whether β-Gal-specific IgG is produced in the absence of normal αβ T cells, *Cre/β-Gal/TCRα*$^{-/-}$ mice were also tested for their ability to produce anti-β-Gal IgG. β-Gal-specific IgG was produced in *Cre/β-Gal/TCR*$^{\alpha-/-}$ mice after OHT administration, although the levels were low (Fig. 6C). These results suggested that γδ T cells played an essential role at least in the initiation of IgG production against the antigen expressed in the epidermis. Switching to IgG, however, was mainly performed by conventional T cells. It seemed that only when γδ T cells were highly activated or a large number of γδ T cells moved to the lymph nodes by sensing abnormal epidermal dysregulation, γδ T cells induced high levels of IgG, especially IgG2a, against newly expressed epidermal antigens (Fig. 3D). It is also possible that the activated γδ T cells interacted with B cells non-specifically. Indeed, we found higher levels of serum IgG in Notch1$^{-/-}$ mice 6–8 months after *Notch1* ablation (data not shown). However, further analysis is required to determine whether the activation of γδ T cells in response to epidermal dysregulation causes autoimmune responses.

Discussion

The prevention of tumor development by tissue tumor immunosurveillance is of great importance. However, most studies have analyzed immune responses against established tumor cells. In an attempt to examine the initiation process of the immune response, a tissue-specific inducible gene-targeting approach was used. In this study, we investigated whether the immune system can sense precancerous dysregulation in the epidermis induced by *Notch1* ablation. Interestingly, epidermal γδ T cells migrated into the cutaneous draining lymph nodes, indicating that epidermal dysregulation caused by a gene mutation prior to tumor development could be sensed by immunosurveillance. In addition, a neo-self-antigen, which is a putative model antigen expressed upon dysregulation, was presented to B cells and antigen-specific IgG production was augmented.

γδ T cells sensed the dysregulation of keratinocytes as early as 1 week after OHT treatment. It was previously shown that up-regulation of Rae-1, an NKG2D ligand, in the epidermis induced γδ T cell activation and αβ T cell infiltration (35). *Notch1* inactivation in keratinocytes might have induced NKG2D ligand expression as aberrant keratinocyte proliferation may cause DNA damage responses. In our system, although the expression of neither the NKG2D ligand *Rae-1* nor *H60* was detected in this early stage, it is possible that different types of NKG2D ligands may have been expressed. As p53 was up-regulated in the *Notch1*-inactivated epidermis, keratinocytes were already affected and expressed some stress-related factors. *S100A8* expression was significantly up-regulated in the *Notch1*-inactivated epidermis, which could indicate aberrant proliferation and cell stress. S100A8 can be induced with various stimuli, including UV irradiation and ionizing radiation. Phorbol myristate acetate (PMA), a tumor-promoting chemical, also up-regulates S100A8 and S100A9 (33) and induces inflammatory factors (36). In the *Notch1*-deleted epidermis, however, expression levels of inflammatory cytokines such as *TNF-α* and *IL-6* were not elevated. These factors are released from keratinocytes and promote inflammation, which is also linked to tumor development (37). Primary keratinocytes treated with PMA express *TNF-α*, *IL-6*, granulocyte macrophage colony-stimulating factor and *S100A8*; however, the induction of these genes, except *S100A8*, depends on nuclear factor κB activation (38), suggesting that the precancerous state induced by *Notch1* ablation is distinct from the classical inflammation-associated tumor model. It is still unknown what molecules expressed by stressed keratinocytes activated epidermal γδ T cells in this model, and future studies will attempt to identify these factors.

Epidermal LCs capture antigens in the skin and migrate to the cutaneous lymph nodes, where they present antigens to

Fig. 5. Migration of γδ T cells into the lymph nodes is promoted in the *Notch1*-inactivated epidermis. (A) An epidermal cell suspension was prepared from control and *Notch1*-inactivated mice 1 week after induction and analyzed by FACS. CD45$^+$ hematopoietic cells were gated and the percentage of each population was calculated. Values are means ± SDs of three independent experiments. *, $P < 0.05$ (Student's *t*-test). (B) Expression of rearranged Vγ5 in cutaneous draining lymph node cells was analyzed by real-time quantitative reverse transcription–PCR. Results were normalized to HPRT mRNA levels. Results represent the means of triplicate samples ± SD. One representative data out of three independent experiments are shown. Age-matched wild-type mice injected with OHT were used as a control. (C) Cutaneous draining lymph node cells were obtained 1 week after induction and stained with antibodies against TCRγδ, B220, TCRβ, CD11c and Vγ5. TCRγδ$^+$ cells in B220$^-$, TCRβ$^-$ and CD11c$^-$ cells were gated and Vγ5$^+$ cells were analyzed. (D) Epidermal cells, either freshly isolated or cultured for 48 h in the presence or absence of 0.5 μM ionomycin, were stained with antibodies against CCR7, CD25 and TCRγδ and analyzed by FACS. Dead cells were removed by Ficoll-Paque centrifugation, TCRγδ$^+$ cells were gated and analyzed for CD25 and CCR7 expression. One representative analysis out of four independent experiments is shown. (E) Epidermal cells (1×10^7 ml^{-1}) were put into a transwell chamber (100 μl) that was placed in a well filled with 600 μl medium with or without 100 nM CCL21. After 48 h, the cells that had migrated out of the chamber were counted. Results represent mean ± SD. ($n = 4$) (F) TCRγδ and I-A expression on freshly isolated epidermal cells and cells that had migrated into the CCL21-containing medium is shown. Migrated cells contained small and large cell populations, which were gated on separately. Data in (C, D and F) are representative of four independent experiments.

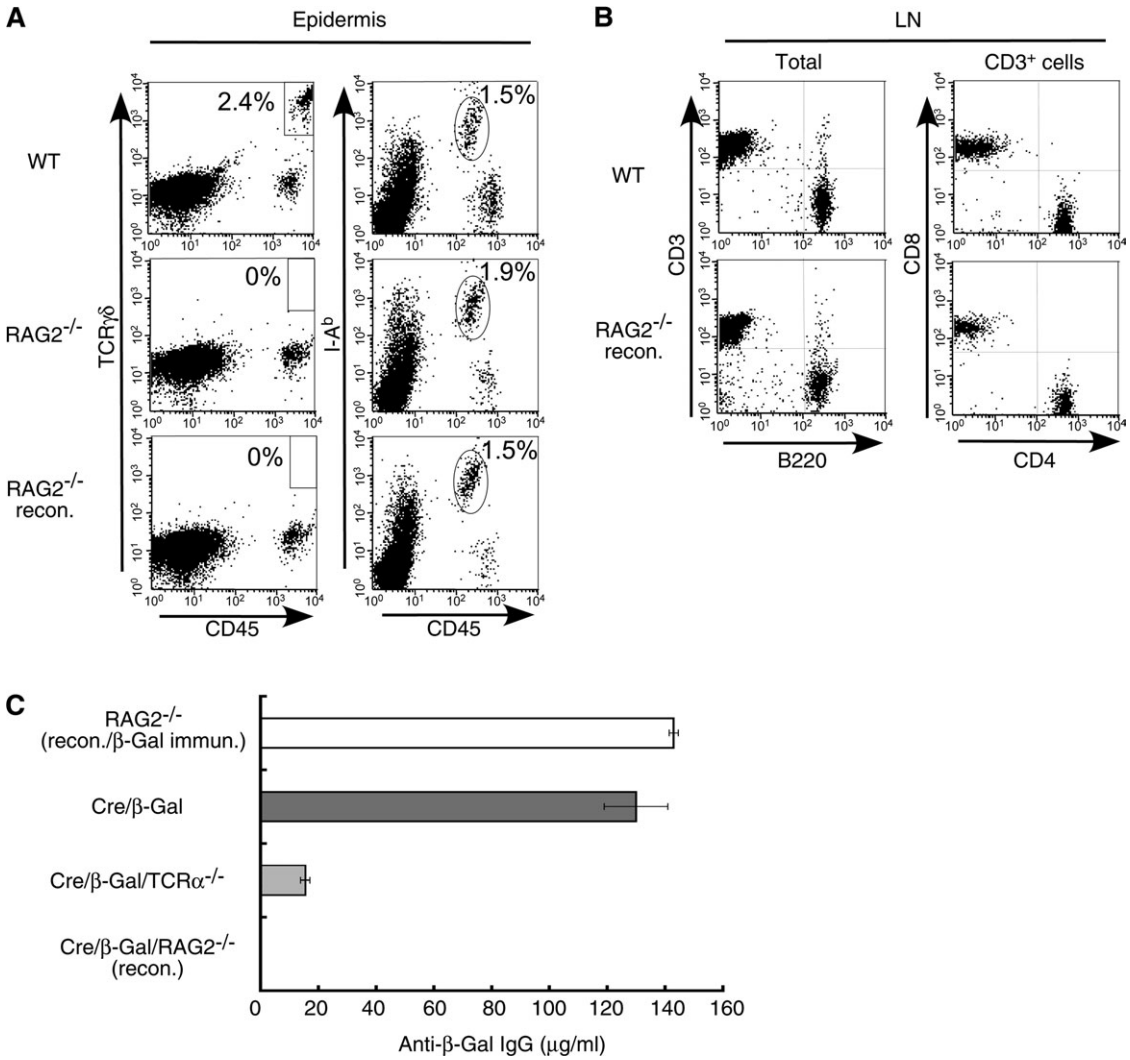


Fig. 6. Requirement of γδ T cells for anti-β-Gal IgG production. (A) Epidermal cells from wild-type mice, *RAG2*$^{-/-}$ and *RAG2*$^{-/-}$ mice that had received 1×10^8 wild-type splenocytes (*RAG2*$^{-/-}$ reconstituted) were stained with anti-I-A or anti-TCRγδ together with anti-CD45 as a marker of hematopoietic cells. (B) Cutaneous draining lymph node cells from the same mice analyzed in (A) were stained with anti-B220 and anti-CD3, and CD3$^+$ cells were further analyzed for CD4 and CD8 expression. (C) *RAG2*$^{-/-}$ mice and mice carrying the *K14-Cre-ER*^{T2}*/CAG-CAT-Z* transgenes (*Cre/β-Gal/RAG2*$^{-/-}$) were reconstituted with 1×10^8 wild-type splenocytes. Two weeks later, *Cre/β-Gal*, reconstituted *Cre/β-Gal/RAG2*$^{-/-}$ and *Cre/β-Gal/TCRα*$^{-/-}$ mice were injected with OHT. As a control, *RAG2*$^{-/-}$ mice reconstituted with splenocytes were immunized with β-Gal. Serum levels of anti-β-Gal IgG were analyzed 3 weeks after induction. Data from a representative analysis out of three independent experiments are shown.

T cells. At steady state, however, dendritic cells are usually tolerogenic when they present antigens (14). Similarly, in our model in which β-Gal expression was induced in keratinocytes upon OHT administration, β-Gal-specific T cell responses were not detected even though β-Gal was a non-self-antigen in these mice. One explanation why these mice were tolerant to β-Gal might be because β-Gal was likely expressed in the thymus after OHT administration, as the *K14* promoter that drives *Cre-ER*^{T2} is active in thymic epithelial cells. However, it has been shown that mice expressing both an antigen driven by the *K14* promoter and a TCR recognizing the antigen develop severe autoimmune disease (39), suggesting that tolerance to β-Gal expressed under the control of the *K14* promoter might not be induced in the thymus. Moreover, self-antigen-specific CD8$^+$ T cells are activated by LCs at steady state when antigen-specific CD8$^+$ T cells are transferred into recipient mice that express the antigen in keratinocytes (40), which argues against the induction of tolerance by LCs at steady state. However, antigen-specific T cell activation in the normal repertoire of naive T cells and not in T cells obtained from TCR transgenic mice may require additional signals to overcome regulatory mechanisms, although LCs have a mature DC phenotype and express CD80/86 in the lymph nodes. In β-Gal$^+$/Notch1$^{-/-}$ mice, which have dysregulated epidermal cells even though stronger responses in the ventral part of the skin were observed, LCs seemed to remain tolerogenic. The lack of inflammatory cytokines, such as TNF-α, could be the main reason why LCs did not activate β-Gal-specific T cell responses.

An interesting finding in this study was that IgG specific for β-Gal induced in keratinocytes was produced in the absence of obvious β-Gal-specific T cell activation. This result suggested that a newly expressed antigen in the epidermis was immunogenic and could induce IgG production. At steady state, there were some γδ T cells expressing Vγ5 in the cutaneous draining lymph nodes of normal mice. It is not clear whether these cells homed from the thymus or migrated out of the epidermis. However, since these cells displayed an activated phenotype, they are likely not naive cells. In the experiment inducing β-Gal in *TCRα*$^{-/-}$ mice, anti-β-Gal IgG was produced although the levels were much lower than those in wild-type β-Gal^{+} mice. We consider that some γδ T cells traffic from the epidermis to the cutaneous draining lymph nodes at steady state and that these γδ T cells contribute to IgG production against epidermal antigens. This idea was supported by the experiment using *RAG2*$^{-/-}$ mice reconstituted with splenocytes. These mice have no epidermal γδ T cells and failed to produce anti-β-Gal IgG. This suggests that during a keratinocyte emergency, γδ T cells in the epidermis are activated and extensively migrate into the lymph nodes, leading to enhanced production of IgG specific for the epidermal antigen. Stimulation of cutaneous draining lymph node cells *in vitro* with anti-CD3 plus anti-CD28 from β-Gal-expressing mice induces prompt expression of *IFN-γ*, which was much higher in β-Gal^{+}/Notch1$^{-/-}$ than in β-Gal^{+} mice. There were more Vγ5^{+}γδ T cells in the lymph nodes in Notch1$^{-/-}$ mice, suggesting that γδ T cells that migrated from the epidermis secreted IFN-γ that induced class switching to IgG2a. In fact, β-Gal induction together with *Notch1* deletion resulted in high levels of β-Gal-specific IgG2a. In mice induced only to express β-Gal, IgG1 levels were higher than IgG2a levels among the anti-β-Gal isotypes, suggesting that there was also some conventional T cell help although we could not detect β-Gal-specific T cell activation. It is still not clear whether γδ T cells could augment only the production of IgG specific for neo-antigens, as they may not discriminate self- and non-self-antigens. We suspected that the total IgG2a levels in *Notch1*-inactivated mice were also elevated after the induction. However, although total IgG levels were high, we did not find elevated IgG2a levels (data not shown). It is therefore possible that γδ T cell activation after *Notch1* deletion was transient and that these cells were only involved in the initiation process. In addition, self-reactive B cells are usually suppressed by another regulatory mechanism.

The involvement of γδ T cells in B cell class switching was previously shown (41, 42). *TCRα*$^{-/-}$ mice infected with a vaccinia virus expressing the vesicular stomatitis virus (VSV) glycoprotein were able to induce VSV-specific IgG class switching (34). As has been shown in human peripheral γδ T cells (20), the lymph node homing receptor CCR7 was induced in epidermal γδ T cells upon activation *in vitro*. Human γδ T cells are also able to function as professional APC and can be induced to express MHC class II molecules (43). It is possible that murine γδ T cells could also capture antigens in the epidermis and carry them into the draining lymph nodes. Alternatively, β-Gal expressed in keratinocytes was taken up by LCs and brought to the draining lymph nodes, and co-migrating γδ T cells helped to induce β-Gal-specific B cell activation.

In tumor immunosurveillance, both innate and adaptive immunity help to prevent tumor development. Activation of γδ T cells that express NKG2D have been shown to suppress a cutaneous malignancy in the epithelia (19). In the *Notch1*-inactivated epidermis, stress-related molecules expressed by dysregulated keratinocytes initiated γδ T cell activation. These activated γδ T cells migrated into the draining lymph nodes and mediated the induction of adaptive immune responses. It is not clear whether antibodies against the antigen expressed in precancerous cells can target to kill them. In our model, antigen-specific T cell responses, which are thought to play a central role in tumor immunosurveillance, did not seem to be activated. The sporadic tumor model that expresses an immunogenic antigen induces antigen-specific T cells and antigen-specific IgG production (44); however, tolerance is induced in T cells by tumor cells. In the precancerous state, however, tolerance may not be induced yet. It will be interesting to determine whether antigen-specific T cells are activated by inducing additional inflammatory signals, which were not observed in the *Notch1*-deleted dysregulated epidermis. Furthermore, the transition from the precancerous state to tumor development may alter T cell responsiveness. Since tumor formation in *Notch1*-inactivated mice was very slow, we are now establishing a model to induce the expression of a constitutively active oncogene in addition to *Notch1* inactivation, where tumor formation occurs rapidly. These mice will further elucidate the roles of γδ T cells and other T cells in preventing tumor development.

Supplementary material

Supplementary data are available at *International Immunology* Online.

Funding

Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Acknowledgements

We thank Dr S. Koyasu and Dr T. Azuma for helpful discussions and Dr H. Kikutani for critically reading the manuscript.

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