

Crucial Role of Phospholipase C ϵ in Induction of Local Skin Inflammatory Reactions in the Elicitation Stage of Allergic Contact Hypersensitivity

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Phospholipase C ϵ (PLC ϵ) is an effector of Ras/Rap small GTPases. We previously demonstrated that PLC ϵ plays a crucial role in development of phorbol ester-induced skin inflammation, which is intimately involved in the promotion of skin carcinogenesis. In this study, we have examined its role in local skin inflammatory reactions during development of contact hypersensitivity toward a hapten 2,4-dinitrofluorobenzene (DNFB). PLC $\epsilon^{+/+}$ and PLC $\epsilon^{-/-}$ mice were sensitized with DNFB, followed by a DNFB challenge on the ears. PLC $\epsilon^{-/-}$ mice exhibited substantially attenuated inflammatory reactions compared with PLC $\epsilon^{+/+}$ mice as shown by suppression of ear swelling, neutrophil infiltration, and proinflammatory cytokine production. In contrast, the extent and kinetics of CD4⁺ T cell infiltration showed no difference depending on the PLC ϵ background. Adoptive transfer of CD4⁺ T cells from the sensitized mice to naive mice between PLC $\epsilon^{+/+}$ and PLC $\epsilon^{-/-}$ backgrounds indicated that PLC ϵ exerts its function in cells other than CD4⁺ T cells, presumably fibroblasts or keratinocytes of the skin, to augment inflammatory reactions during the elicitation stage of contact hypersensitivity. Moreover, dermal fibroblasts and epidermal keratinocytes cultured from the skin expressed proinflammatory cytokines in a PLC ϵ -dependent manner on stimulation with T cell-derived cytokines such as IL-17, IFN- γ , TNF- α , and IL-4. These results indicate that PLC ϵ plays a crucial role in induction of proinflammatory cytokine expression in fibroblasts and keratinocytes at the challenged sites, where infiltrated CD4⁺ T cells produce their intrinsic cytokines, thereby augmenting the local inflammatory reactions. *The Journal of Immunology*, 2010, 184: 000–000.

Contact hypersensitivity (CHS) is a form of T cell-mediated cutaneous immune and inflammatory reactions induced by repeated exposure to the chemical substance, hapten. The development of CHS is composed of two stages: induction/sensitization and elicitation (1). In the elicitation/sensitization stage, a hapten penetrates the skin, where it combines with protein present in the epidermis to form a haptenated peptide. The haptenated peptide can then function as a complete Ag and is subsequently transported to the local draining lymph nodes, where an immune response is stimulated. The elicitation stage can be initiated by a subsequent challenge of the sensitized animal with the same hapten. The haptenated peptide is presented by APC, such as epidermal Langerhans cells, and hapten-specific T cells are selectively recruited to the extravascular space at the challenged sites within

2 h (2). The recruited T cells start to produce cytokines, such as IL-17 and IFN- γ , which stimulate skin cells, including keratinocytes and fibroblasts, to express cytokines and cell adhesion molecules that facilitate leukocyte recruitment (3, 4). Through these actions, T cells elicit cutaneous inflammatory reaction phase that peaks after ~24 h (2). The dermatitis in this stage is characterized by edema, itching, redness, pain, rash, etc. Because it can be easily reproduced in animals, CHS serves as a good experimental model for studying allergy and autoimmunity.

Phosphoinositide-specific phospholipase C (PLC) plays fundamental roles in intracellular signaling by hydrolyzing phosphatidylinositol 4,5-bisphosphate to 1,2-diacylglycerol and inositol 1,4,5-trisphosphate. 1,2-diacylglycerol binds to a variety of the target proteins, including protein kinase C (PKC) isozymes and Ras guanyl nucleotide-releasing proteins (RasGRPs), and regulates their activities. Inositol 1,4,5-trisphosphate induces the release of calcium ion from the intracellular stores, leading to an increase in the cytosolic-free calcium concentration. At least 13 mammalian PLC isoforms have been identified and organized into six classes (β , γ , δ , ϵ , ζ , and η) (5, 6). PLC ϵ was identified as an effector of Ras-family small GTPases: Ras, Rap1, and Rap2 (7–10). Similar to other effectors, such as cRaf-1 and B-Raf, PLC ϵ is activated by direct association with the GTP-bound form of the small GTPases through its Ras/Rap-associating domain (7–10). Subsequent studies have demonstrated that PLC ϵ is also activated by another small GTPase RhoA and heterotrimeric G proteins α_{12} and $\beta_1\gamma_2$ subunits (10).

We showed that PLC $\epsilon^{-/-}$ mice, in which PLC ϵ was catalytically inactivated by gene targeting, exhibited marked resistance to tumor formation in the two-stage skin chemical carcinogenesis protocol using 7,12-dimethylbenz(a)anthracene as an initiator and a phorbol ester PMA as a promoter (11). Subsequent studies showed that PMA-induced skin inflammation, which is intimately involved in tumor promotion, was attenuated in PLC $\epsilon^{-/-}$ mice (12). Using primary-cultured dermal fibroblasts prepared from PLC $\epsilon^{-/-}$ mice,

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Abbreviations used in this paper: CHS, contact hypersensitivity; DNFB, 2,4-dinitrofluorobenzene; PKC, protein kinase C; PLC ϵ , phospholipase C ϵ ; QRT-PCR, quantitative RT-PCR; RasGRP, Ras guanyl nucleotide-releasing protein; UD, below detection limit.

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we showed that PMA treatment gives rise to the activation of PLC ϵ , which is mediated by Rap1 activation induced by the direct activation of two PMA targets, RasGRP3 and PKC, thereby inducing the expression of proinflammatory molecules, such as IL-1 α (12). Recently, we have demonstrated that PLC $\epsilon^{-/-}$ mice also exhibit marked resistance to Apc^{Min}-dependent intestinal tumorigenesis, which appears to be closely associated with the attenuation of tumor-associated inflammation (13). These results suggest a crucial role of PLC ϵ in a wide variety of inflammations. However, little is known about how PLC ϵ functions in inflammatory reactions.

In this study, we use a mouse CHS model in which allergic dermatitis is induced by a hapten 2,4-dinitro-1-fluorobenzene (DNFB). Substantial attenuation of the inflammatory reactions in the elicitation stage of CHS is observed in PLC $\epsilon^{-/-}$ mice. Comparison of the infiltration of CD4⁺ T cells and the expression of various proinflammatory cytokines between PLC $\epsilon^{+/+}$ and PLC $\epsilon^{-/-}$ mice, combined with the adoptive transfer of CD4⁺ T cells, indicate that PLC ϵ functions in efficient production of proinflammatory cytokines from dermal fibroblast and epidermal keratinocytes in response to T cell-derived cytokines, such as IL-17, IFN- γ , TNF- α , and IL-4.

Materials and Methods

Animals

Mice homozygous for the inactivated PLC ϵ allele (PLC $\epsilon^{-/-}$), created by in-frame deletion of an exon coding for the catalytic X domain, were generated as described previously (11, 14) and backcrossed to C57BL/6J mice (CLEA Japan, Tokyo, Japan) for at least eight generations. Genotyping of the mutant animals was performed by PCR using allele-specific primers (11, 14). The use and care of the animals were reviewed and approved by the Institutional Animal Committee of Kobe University Graduate School of Medicine.

Abs, ligands, and chemicals

Primary Abs used were as follows: anti-CD4 (550280, BD Pharmingen, San Diego, CA), anti-Gr-1 (MAB1037, R&D Systems, Minneapolis, MN), anti-CXCL-1 (AF-453-NA, R&D Systems), anti-CXCL-2 (AF-452-NA, R&D Systems), anti-CCL-20 (AF760, R&D Systems), anti-IL-1 α (AF-400-NA, R&D Systems), anti-IL-1 β (AF-401-NA, R&D Systems), and anti-PLC γ 1 (sc-81, Santa Cruz Biotechnology, Santa Cruz, CA). Anti-PLC ϵ Ab raised against the C terminus of mouse PLC ϵ was described (15). Secondary Abs labeled with Alexa dye were purchased from Invitrogen (Carlsbad, CA). HRP-conjugated secondary Abs were purchased from GE Healthcare (Buckinghamshire, U.K.). Ligands and chemicals used were TNF- α (315-01, PeproTech, Rocky Hill, NJ), IFN- γ (315-05, PeproTech), IL-4 (112-40-134S, AMS Biotechnology, Abingdon, Oxford, U.K.), IL-17 (421-ML/CF, R&D Systems), DNFB (D1529, Sigma-Aldrich, St. Louis, MO), and PMA (P-8139, Sigma-Aldrich).

Induction of CHS

A mouse was sensitized with DNFB by painting its shaved dorsal skin with 25 μ l 0.4% (w/v) DNFB dissolved in acetone. Five days later, 20 μ l 0.2% (w/v) DNFB was applied to the backside of the right ear. The same volume of acetone was applied to the left ear as an unchallenged control. For noninvasive evaluation of the inflammation, ear swelling was calculated by subtracting the thickness of the left ear from that of the right ear after measurement with a pair of calipers (MDC-25MJ, Mitutoyo, Kawasaki, Japan) as described (12).

Total WBC counting

Blood samples (30 μ l) were collected from the retro-orbital venous plexus of 8-wk-old male mice ($n = 5$ for each PLC ϵ genotype) and diluted in buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) for hemolysis of erythrocytes. Subsequently, cells were collected by centrifugation, resuspended in 300 μ l PBS, loaded on a hematocytometer, and counted as WBCs under a microscope.

Adoptive transfer of CD4⁺ T cells

CD4⁺ T cells were purified from the spleen of the sensitized animal at 5 d after DNFB sensitization by using mouse CD4⁺ T isolation kit (130-090-860, Miltenyi Biotec, Bergisch Gladbach, Germany) and AutoMACS (Miltenyi Biotec). The purity of the recovered CD4⁺ T cells was estimated >95% by

the immunostaining with anti-CD4 Ab (data not shown). The purified CD4⁺ T cells (5×10^6 cells) were suspended in 0.2 ml sterilized PBS and injected i.v. into recipient mice. One hour later, the recipients' ears were challenged with DNFB or acetone and subjected to the evaluation of the response as described previously.

Histology and immunostaining

Tissues were collected from sacrificed animals, fixed in 4% paraformaldehyde, embedded in paraffin or OCT compound, and sectioned as described previously (12). The sections were subjected to H&E staining or immunostaining. For immunostaining, the sections were fixed again in 4% paraformaldehyde (for anti-CD4, anti-PLC ϵ , anti-PLC γ 1, and anti-Gr-1 immunostaining) or Zamboni's Fixative (Muto Pure Chemicals, Tokyo, Japan) for 30 min, washed, and treated with blocking solutions containing IgG. Probing with primary Abs and fluorescent active Alexa-labeled secondary Abs (Invitrogen), nuclear staining with DAPI, and observation of the fluorescence were performed as described (12–15).

Identification and enumeration of total leukocytes, neutrophils, and CD4⁺ T cells on tissue sections

Leukocytes and neutrophils were identified on H&E-stained skin sections (Supplemental Fig. 1). Particularly, pink-stained leukocytes with nuclei definitely divided into two to five lobes were identified as neutrophils. The identification of neutrophils was performed also with immunostaining using anti-Gr-1 Ab (Supplemental Fig. 1). CD4⁺ T cells were identified by immunostaining with anti-CD4 Ab. Cells present in the dermal layer of the ear auricles were counted on three randomly-chosen areas (at least 1 mm² in size) of each section. Three animals of each group were examined.

RT-PCR

Total cellular RNAs were isolated from cultured cells with Trizol (Invitrogen) and from tissues with RNeasy Mini kit (74134, Qiagen). cDNA synthesis and RT-PCR were performed as described previously (12). Real-time quantitative RT-PCR (QRT-PCR) was carried out using SYBR Premix Ex Taq II kit (Takara Bio, Kyoto, Japan) with Thermal Cycler Dice Real Time System (Takara Bio) as described (13). Relative mRNA levels were determined by the comparative Ct method, followed by normalization with the β -actin mRNA level in each cDNA sample. The sequences of the primers used for RT-PCR and QRT-PCR are listed in Table I (12, 13, 16, 17).

Quantification of cytokines in the culture supernatant by ELISA

Supernatant was collected from dermal fibroblast culture after stimulation with cytokines for 12 h and subjected to quantification of various cytokines by using ELISA kits purchased from R&D Systems (MLA00 for IL-1 α , MLB00B for IL-1 β , MM200 for CXCL-2, and MCC200 for CCL-20).

Primary cultures of epidermal keratinocytes and dermal fibroblasts

Primary culture was performed as described previously (12) with minor modifications. Briefly, epidermal keratinocytes were isolated from the dorsal skin of newborn mice at postnatal day 1 (P1) and seeded with Defined Keratinocyte-SFM supplemented with growth factors (10744-019, Invitrogen) onto the plastic plates coated with type I-A collagen (Nitta gelatin, Osaka, Japan). Keratinocyte differentiation was induced by exposing cells to the media containing 0.5 mM Ca²⁺ for 2 d. The differentiation was monitored by assessment of the expression of differentiation-specific markers as well as the morphology. Dermal fibroblasts were isolated from the dorsal skin of newborn mice at P1–3 and cultured on ordinary culture plates in DMEM supplemented with 10% FBS.

Western blot analysis

Preparation of cell lysates, determination of protein concentrations, SDS-PAGE, and immunoblotting were performed as described previously (12, 13).

Measurement of cytosolic-free calcium concentration

Cytosolic-free calcium concentration in cultured dermal fibroblasts was monitored in cells loaded with Fura-2 AM (Nacalai Tesque, Kyoto, Japan) as described previously (12). Using F-4500 fluorometer (Hitachi, Tokyo, Japan), changes in the fluorescence intensities of the emissions were measured at 510 nm after excitation at 340 and 380 nm. Results are expressed as relative concentrations of cytosolic-free calcium calculated as described (18).

Assessment of the cytokine-inducing activity of the conditioned medium from IL-17-stimulated cells

The conditioned medium from primary-cultured PLC $\epsilon^{+/+}$ dermal fibroblasts stimulated with 10 ng/ml IL-17 for 12 h was added to fibroblasts. At 0, 3, and 6 h after the addition of the conditioned medium, mRNA levels of various cytokine genes were assessed by QRT-PCR.

Statistical analysis

Values are expressed as the means $\pm$ SD. Unpaired Student *t* test was performed for determination of *p*-values using Graphpad Prism software (Graphpad Software). In case the *p*-values were <0.05 , differences were considered to be statistically significant.

Results

Attenuation of CHS reactions in PLC $\epsilon^{-/-}$ mice

To study the role of PLC ϵ in CHS, PLC $\epsilon^{+/+}$ and PLC $\epsilon^{-/-}$ mice were sensitized by painting their dorsal skins with DNFB. Five days later, they were challenged on the ears with DNFB, and the inflammatory reactions were assessed on their ear sections at 24 h after the DNFB challenge (Fig. 1A and Supplemental Fig. 2). H&E staining of the sections indicated that the ears of PLC $\epsilon^{+/+}$ mice showed development of strong inflammatory reactions at the challenged sites, which were characterized by marked spongiosis and extensive granulocyte infiltration in the swollen dermis. In contrast, such reactions were substantially attenuated in the ears of PLC $\epsilon^{-/-}$ mice. Examination of the time course of ear swelling indicated that the ear thickness sharply increased until 12 h after the challenge without noticeable difference depending on the PLC ϵ genetic background (Fig. 1B). Thereafter, substantial difference developed depending on the PLC ϵ background: the ear thickness continuously increased until 48 h after the challenge in PLC $\epsilon^{+/+}$ mice, whereas it exhibited substantial reduction and reached a plateau around 24–36 h in PLC $\epsilon^{-/-}$ mice (Fig. 1B). As the patterns of the ear swelling were found gender-independent (Fig. 1B), additional studies were carried out using male animals

only. Next, the time course of the leukocyte infiltration was examined on the H&E-stained ear sections (Fig. 1C). After the DNFB challenge, the number of overall leukocytes present in the challenged site showed a robust increase in a PLC ϵ -independent manner until 6 h. Thereafter, it increased continuously up to 48 h. By contrast, in PLC $\epsilon^{-/-}$ mice, the increase was substantially blunted starting from 12 h and thereafter. We paid particular attention on neutrophils because they play a crucial role in DNFB-induced CHS (19). As examined on the H&E-stained ear sections, neutrophil infiltration was substantially attenuated on the PLC $\epsilon^{-/-}$ background (Fig. 1C), which was confirmed by immunostaining for Gr-1 (Supplemental Fig. 1). These results suggested the involvement of PLC ϵ in the middle to late phases of the elicitation stage of CHS, starting around 12 h after DNFB challenge.

Involvement of PLC ϵ in upregulation of proinflammatory cytokine expression

The circulating WBC counts were not affected by the PLC ϵ background [$(32.4 \pm 4.0) \times 10^2$ cells/mm³ in PLC $\epsilon^{+/+}$ mice versus $(34.0 \pm 5.3) \times 10^2$ cells/mm³ in PLC $\epsilon^{-/-}$ mice; *p* = 0.382 (*n* = 5 per group)]. In addition, the PLC ϵ background did not seem to intrinsically influence the function of Gr-1-positive granulocytes because granulocytes do not express a detectable amount of PLC ϵ (12). Thus, the suppression of neutrophil infiltration in PLC $\epsilon^{-/-}$ mice is likely to be accounted for by attenuated expression of proinflammatory cytokines in the elicitation stage. To test this assumption, the levels of cytokine mRNAs at the DNFB-challenged sites were quantified by QRT-PCR (Table I) at the different phases of the elicitation stage, namely, 3 h and 24 h after the DNFB challenge representing the early phase and the late phase, respectively (Fig. 2). The level of IL-1 α , whose PLC ϵ -dependent expression had been observed in PMA-induced skin inflammation (12), exhibited significant increase at 3 h, followed by marked increase at 24 h in a PLC ϵ -dependent manner. IL-1 β showed a similar expression pattern. Chemokines, CXCL-2, CXCL-1, and CCL-20,

Table I. The primers used for RT-PCR and QRT-PCR analyses

Gene	Forward Primer (5'–3')	Reverse Primer (5'–3')	Purposes	References
PLC ϵ^a	gaaagctgggtctcctatggc	tgccctctagaagagaaccgg	RT-PCR	12
PLC ϵ^b	gctttgaaacgaggatctgcacatcttcagctgc	ggctttgcataaggctctgctgaattacatcc	RT-PCR	16
PLC γ 1	gacatcacctacgggcagtt	cacacgtctgttctcttggga	RT-PCR	
GAPDH	gtgaaggtcgggtgtgaacggattt	cacagctcttctgagtgccagtgat	RT-PCR	12
β -actin	ctacaatgagctgctgtgtgg	caacgtcacacttcatgatgg	RT-PCR	12
IL-4	acggcacagagctattgatg	atgggtggctcagtagctacga	QRT-PCR	
CD4	ttcagagcacagctatcacg	tcactcagtagacactgccca	RT-PCR	
CXCL-1	accacaaaccgaagtcatagc	tggggacacaccttttagcatc	QRT-PCR	13
CXCL-2	agtttgccttgaccctgaag	cttttggttcttccgcttgagg	QRT-PCR	13
TNF- α	tgatcgggtcccaaagg	ggctctgggcatagaactga	QRT-PCR	
β -actin	atgaagatcaagatcattgtctctc	acatctgctggaaggtggacag	QRT-PCR	13
IL-17A	tccagaaggccctcagacta	ctcgacctgaaagtgaagg	QRT-PCR	
IFN- γ	actggcaaaaggatggtgac	tgagctcattgaatgcttg	QRT-PCR	
IL-22	tccgaggagtcagtgctaaa	agaacgtcttccagggtgaa	QRT-PCR	17
IL-1 α	gtccataaccatgatctgg	caagtggtgctgagatagtg	QRT-PCR	
IL-1 β	caccctgcagctggagagt	gacaaaccgcttttccatcttc	QRT-PCR	
CCL-20	caccagttctgctttggat	cgactgttgctctctgtaca	QRT-PCR	
CD4	aagaactgggttcggcatgac	tccctctgcaaagttgagtg	QRT-PCR	
IL-1 α	tggcaagttcctgacttgtttg	caggctcatttaaccaagtggtgt	RT-PCR	12
IL-1 β	atggcaactgttctgactcaact	caggacaggtatagattctttccttt	RT-PCR	12
CXCL-1	gcttggtcagtttaaagatggtaggc	cggtgtgaccatacaatatgaagacg	RT-PCR	12
CXCL-2	ctgccgtctcctcagtgctgactg	gccttgctcttggctcagtatcttttg	RT-PCR	12
Keratin 1	agcaaggtggaagctgagac	gggcatctttgagtgctttc	RT-PCR	
Keratin 5	gagctggctctcaaagatgc	tgactgggtccaaactccttc	RT-PCR	
Keratin 10	tcaccacagaaatcgacacg	cggagatctggctttgaaac	RT-PCR	

^aPrimer set A used in Fig. 4.

^bPrimer set B used in Fig. 4.

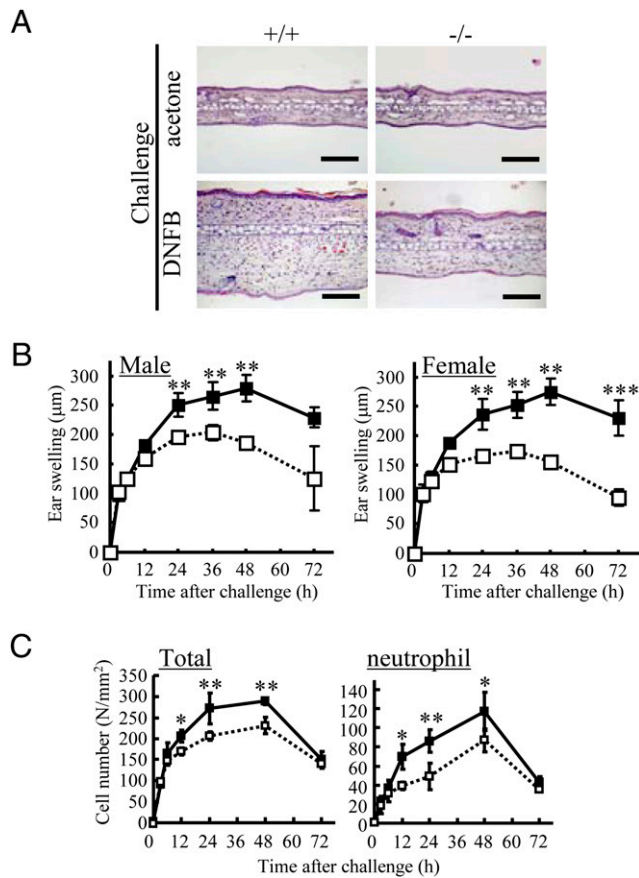


FIGURE 1. Comparison of DNFB-induced CHS reactions between PLC $\epsilon^{+/+}$ and PLC $\epsilon^{-/-}$ mice. **A**, Ear swelling after DNFB challenge. Ears of the sensitized male mice were painted with DNFB or acetone vehicle. Twenty-four hours later, the ear auricles of PLC $\epsilon^{+/+}$ (+/+) and PLC $\epsilon^{-/-}$ (-/-) mice were collected and their H&E-stained sections were prepared. Bars, 200 μ m. Higher magnification pictures are provided in Supplemental Fig. 2. **B**, Time course of the ear swelling after the DNFB challenge. The extents of inflammation was noninvasively assessed in PLC $\epsilon^{+/+}$ (filled symbols) and PLC $\epsilon^{-/-}$ (open symbols) mice by measuring the ear thickness. Data are expressed as the means $\pm$ SD obtained from three animals of each group. ** p < 0.01; *** p < 0.001 between PLC $\epsilon^{+/+}$ and PLC $\epsilon^{-/-}$ mice. **C**, Time course of leukocyte infiltration. Total leukocytes and neutrophils were counted on the H&E-stained ear sections of three each of PLC $\epsilon^{+/+}$ (filled symbols) or PLC $\epsilon^{-/-}$ (open symbols) males. Data are expressed as the means $\pm$ SD (n = 3 per group). * p < 0.05; ** p < 0.01 between PLC $\epsilon^{+/+}$ and PLC $\epsilon^{-/-}$ mice.

showed significant elevation at 3 h, though, in a PLC ϵ -independent manner, followed by marked elevation at 24 h in a PLC ϵ -dependent manner. PLC ϵ -dependent expression of these five cytokines in situ was demonstrated by immunostaining of the DNFB-challenged ears; they showed robust expression at 24 h after the challenge in both epidermal keratinocytes and dermal fibroblasts of PLC $\epsilon^{+/+}$ mice, which was substantially attenuated in those of PLC $\epsilon^{-/-}$ mice (Fig. 3, Supplemental Fig. 3). These results suggested that the suppression of neutrophil infiltration in PLC $\epsilon^{-/-}$ mice could be accounted for by reduced production of these proinflammatory cytokines. Because production of these cytokines after T cell infiltration is triggered by T cell-derived cytokines, we next examined the expression of such cytokines, IL-17A, IFN- γ , IL-4, and TNF- α , which had been implicated in the development of CHS (20–22) (Fig. 2). The levels of IL-17A, IFN- γ , and IL-4 exhibited a small but significant increase at 3 h, followed by a marked increase at 24 h. The increase of these cytokine levels was found independent of the PLC ϵ background at the both time points,

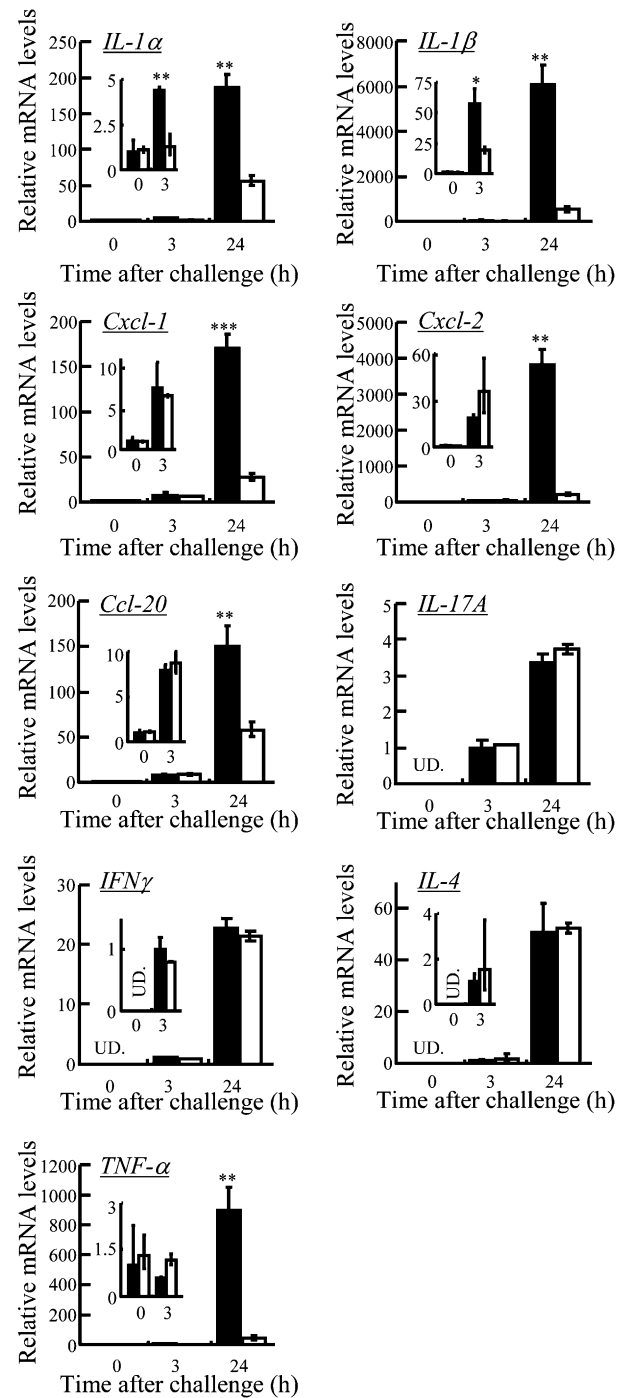


FIGURE 2. Effects of the PLC ϵ background on induction of proinflammatory cytokines. The levels of mRNAs encoding the indicated cytokines were measured in the ears of three each of PLC $\epsilon^{+/+}$ (black bars) or PLC $\epsilon^{-/-}$ (white bars) mice by QRT-PCR. Relative mRNA levels of the indicated cytokines normalized to the corresponding mRNA levels in PLC $\epsilon^{+/+}$ mice at 0 h (for IL-1 α , IL-1 β , CXCL-1, CXCL-2, CCL-20, and TNF- α) or at 3 h (for IL-17A, IFN- γ , and IL-4) are shown as the means $\pm$ SD of triplicate determinations. In case the increase of the mRNA level is significant, p -values between PLC $\epsilon^{+/+}$ and PLC $\epsilon^{-/-}$ mice are indicated (* p < 0.05; ** p < 0.01; *** p < 0.001). UD, below detection limit.

which apparently contradicts with the previously reported data that PLC ϵ was involved in Ras-dependent upregulation of cytokine genes in CD4⁺ T cells (16). Our results were further supported by an analysis of PLC ϵ expression in CD4⁺ T cells, which demonstrated the absence of PLC ϵ at both the protein and mRNA levels

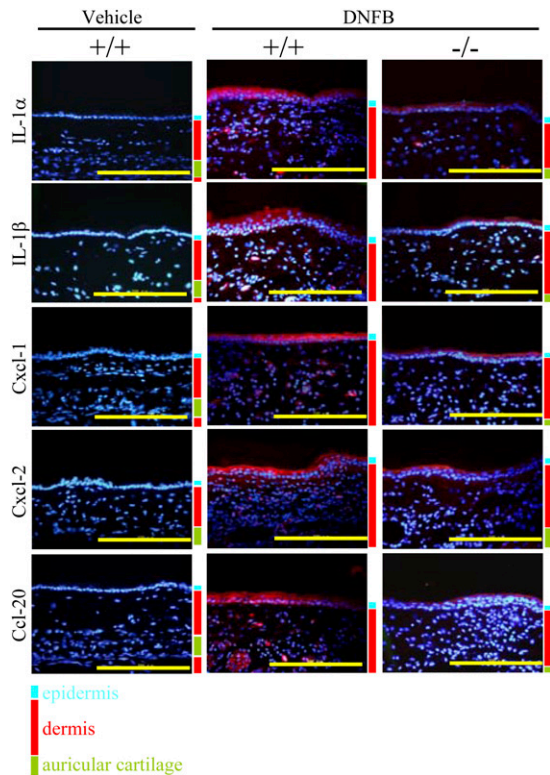


FIGURE 3. Immunostaining analysis of the cytokine production. Cytokines in the ears of $PLC\epsilon^{+/+}$ and $PLC\epsilon^{-/-}$ mice were detected by immunostaining using their specific Abs (red) at 24 h after the challenge with DNFB or acetone vehicle. Bars on the right margins of the pictures denote the epidermis (blue), dermis (red) and auricular cartilage (green). Scale bars, 200 μ m. Higher magnification pictures are provided in Supplemental Fig. 3.

in contrast to the abundant presence of $PLC\gamma 1$ (Fig. 4, Supplemental Figs. 4 and 5). In contrast, $TNF-\alpha$ showed no increase at 3 h, followed by a marked elevation in a $PLC\epsilon$ -dependent manner at 24 h. This $PLC\epsilon$ -dependent increase may be accounted for by the attenuated infiltration of neutrophils (Fig. 1), another species of $TNF-\alpha$ -producing cells (23), in $PLC\epsilon^{-/-}$ mice. IL-22, another T cell-derived cytokine implicated in the pathogenesis of certain types of skin inflammation (24), was not induced at all within 24 h after the DNFB challenge (Supplemental Fig. 6), indicating that this cytokine does not play a role in the elicitation. These results hinted possible involvement of $PLC\epsilon$ in downstream signaling from IL-17A, IFN- γ , $TNF-\alpha$, or IL-4, which is produced by T cells infiltrated into the challenged site.

CD4⁺ T cell function is independent of the $PLC\epsilon$ background

During the development of CHS, IL-17 plays a key role in induction of local tissue inflammation (3, 25). The major targets of this cytokine include fibroblasts and keratinocytes (3, 24–26), where $PLC\epsilon$ is abundantly present (12) (Fig. 4A). Recent studies demonstrated that one of the major producers of IL-17 is a subclass of $CD4^{+}$ Th cells, called Th17 (3). We therefore asked whether the $PLC\epsilon$ background indeed affected the infiltration of $CD4^{+}$ T cells to the DNFB-challenged site (Fig. 5, A–C, Supplemental Fig. 7). The time course of the $CD4^{+}$ T cell infiltration exhibited no difference depending on the $PLC\epsilon$ background throughout the experimental periods ranging from 3 to 72 h after challenge as shown by immunostaining (Fig. 5A, 5B, Supplemental Fig. 6). Also, quantification of the CD4 mRNA levels supported this notion (Fig. 5C). These results indicated that $CD4^{+}$ T cell infiltration was not affected

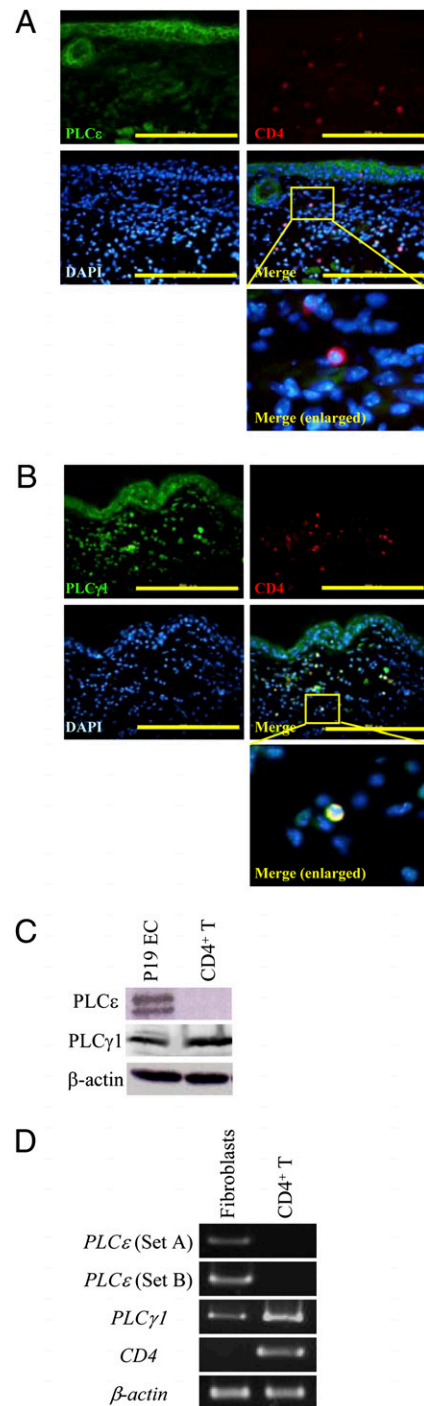
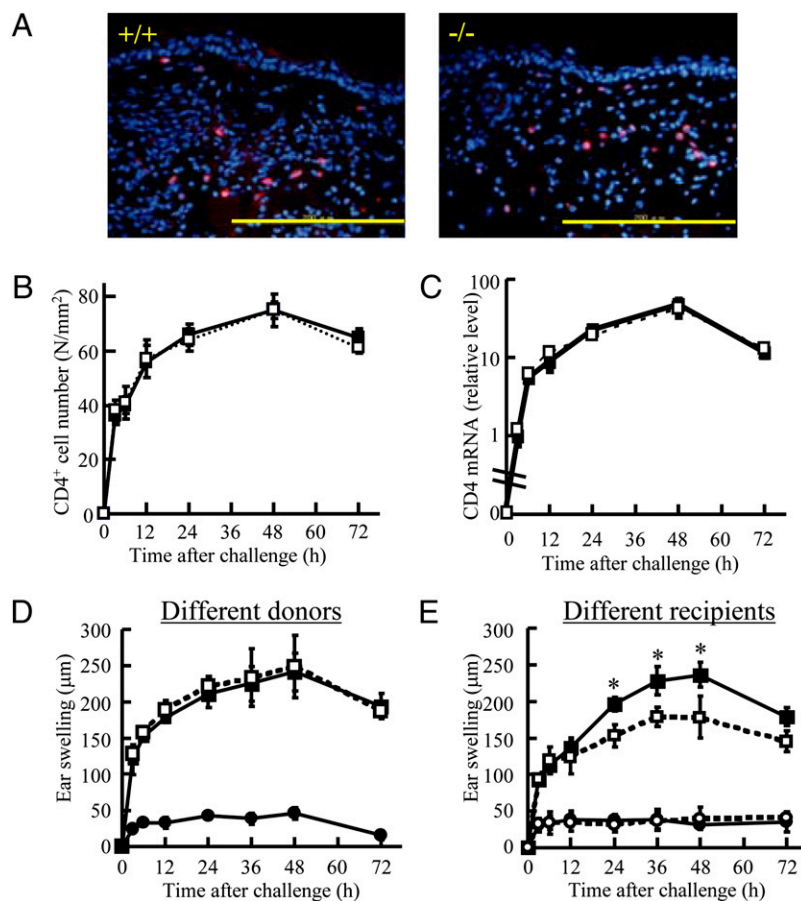


FIGURE 4. Demonstration of the absence of $PLC\epsilon$ in $CD4^{+}$ T cells. **A** and **B**, In situ immunostaining. Cross sections of the ears of a $PLC\epsilon^{+/+}$ mouse at 24 h after the DNFB challenge were subjected to immunostaining for $PLC\epsilon$ (**A**, green) and $PLC\gamma 1$ (**B**, green) as well as for CD4 (red). Nuclei were visualized by DAPI staining. A merged image of all the three staining and its enlargement are also shown. Bars, 200 μ m. Higher magnification pictures are provided in Supplemental Fig. 4. **C**, Western blot analysis. Lysates of purified $CD4^{+}$ T cells ($CD4^{+}$ T) and P19 mouse embryonic carcinoma cells (P19 EC) (30 μ g protein/lane determined by the Bradford assay) were fractionated by SDS-PAGE and subjected to immunoblotting with anti- $PLC\epsilon$ and anti- $PLC\gamma 1$ Abs. Anti- β -actin blot was used as loading control. **D**, RT-PCR analysis. The $PLC\epsilon$ mRNA level was estimated in cultured dermal fibroblasts and purified $CD4^{+}$ T cells by semiquantitative RT-PCR with 35 cycles of amplification. For detection of the $PLC\epsilon$ mRNA, two different primer sets (set A and set B) were used. The result of amplification of β -actin is shown as loading control.

FIGURE 5. Effect of the PLC ϵ background on the activity of CD4 $^{+}$ T cells. **A**, Immunostaining analysis of the CD4 $^{+}$ T cell infiltration. Cross sections of the ears of PLC $\epsilon^{+/+}$ and PLC $\epsilon^{-/-}$ mice at 24 h after the DNFB challenge were subjected to immunostaining for CD4 (red). Nuclei were visualized by DAPI staining (blue). Bars, 200 μ m. Higher magnification pictures are provided in Supplemental Fig. 7. **B**, Time course of the infiltration of CD4 $^{+}$ T cells. CD4 $^{+}$ T cells in the DNFB-challenged skin of PLC $\epsilon^{+/+}$ (filled symbols) and PLC $\epsilon^{-/-}$ (open symbols) mice were counted. Data are expressed as the means $\pm$ SD of triplicate determinations as described in *Materials and Methods*. **C**, Measurement of the CD4 mRNA. The CD4 mRNA level was measured by QRT-PCR in the ear skin of PLC $\epsilon^{+/+}$ (filled symbols) and PLC $\epsilon^{-/-}$ (open symbols) mice. Relative CD4 mRNA levels normalized to the mRNA level in PLC $\epsilon^{+/+}$ mice at 3 h are shown as the means $\pm$ SD of triplicate determinations. **D**, CHS response in PLC $\epsilon^{+/+}$ recipients to which CD4 $^{+}$ T cells were adoptively transferred from mice with different PLC ϵ genotypes. CD4 $^{+}$ T cells purified from DNFB-sensitized mice with the PLC $\epsilon^{+/+}$ (filled square symbols on solid lines) or PLC $\epsilon^{-/-}$ (open square symbols on broken lines) genotype were transferred to recipient naive PLC $\epsilon^{+/+}$ mice. As a negative control, PBS was injected into naive mice (filled circle symbols on solid lines). Data are shown as the means $\pm$ SD ($n = 3$ per group). **E**, CHS response in mice carrying different PLC ϵ genotypes to which CD4 $^{+}$ T cells were adoptively transferred from PLC $\epsilon^{+/+}$ mice. CD4 $^{+}$ T cells purified from sensitized PLC $\epsilon^{+/+}$ mice were transferred to recipient naive mice with the PLC $\epsilon^{+/+}$ (filled square symbols on solid lines) or PLC $\epsilon^{-/-}$ (open square symbols on broken lines) genotype. As a negative control, PBS was injected into recipients PLC $\epsilon^{+/+}$ (filled circle symbols on solid lines) or PLC $\epsilon^{-/-}$ (open circle symbols on broken lines) mice. Data are shown as the means $\pm$ SD ($n = 3$ per group). * $p < 0.05$ between PLC $\epsilon^{+/+}$ and PLC $\epsilon^{-/-}$ recipients.



by the PLC ϵ background, suggesting that PLC ϵ may mainly function in the inflammatory reactions after the T cell infiltration. To test this possibility, we carried out adoptive transfer experiments of CD4 $^{+}$ T cells between PLC $\epsilon^{+/+}$ and PLC $\epsilon^{-/-}$ mice. At first, CD4 $^{+}$ T cells purified from the DNFB-sensitized mice carrying the PLC $\epsilon^{+/+}$ or PLC $\epsilon^{-/-}$ background were adoptively transferred into naive PLC $\epsilon^{+/+}$ mice, followed by DNFB challenge of the recipients' ears. As expected, the PLC ϵ background of the donor mice did not affect the development of the inflammatory reactions (Fig. 5D). In contrast, when CD4 $^{+}$ T cells from the DNFB-sensitized PLC $\epsilon^{+/+}$ mice were transferred into naive mice carrying the PLC $\epsilon^{+/+}$ or PLC $\epsilon^{-/-}$ background, the PLC $\epsilon^{-/-}$ recipient mice developed substantially attenuated inflammatory reactions compared with the PLC $\epsilon^{+/+}$ recipients (Fig. 5E). These results implied that PLC ϵ exerts its function in cells other than CD4 $^{+}$ T cells, presumably, fibroblasts or keratinocytes of the skin, to augment inflammatory reactions during the elicitation stage, but not the sensitization stage, of CHS.

PLC ϵ -dependent induction of proinflammatory cytokines in cultured skin cells by T cell-derived cytokines

The results so far indicated the importance of fibroblasts or keratinocytes of the skin as the cellular site of the PLC ϵ action, which presumably involves expression of proinflammatory cytokines in response to T cell-derived signals. Thus, we examined whether T cell-derived cytokines implicated in CHS, such as IL-17, IFN- γ , TNF- α , and IL-4 (20–22), could induce proinflammatory cytokine expression from fibroblasts or keratinocytes in a PLC ϵ -dependent manner. To this end, primary cultures of fibroblasts (Fig. 6) and keratinocytes (Fig. 7) established from PLC $\epsilon^{+/+}$ and PLC $\epsilon^{-/-}$ mice skins were stimulated by each of the four cytokines for 12 h and analyzed for the production of a variety of proinflammatory cytokines

by QRT-PCR (Figs. 6A, 7B) and ELISA (Figs. 6B, 7C). In fibroblasts, IL-1 α was found to be efficiently induced by IL-17 stimulation, which showed clear dependence on the PLC ϵ background. Likewise, CXCL-2 was induced by IFN- γ stimulation, whereas CCL-20 was induced by TNF- α stimulation, both of which exhibited significant PLC ϵ -dependence. Further, certain combinations of these cytokines yielded more efficient induction of IL-1 α and CXCL-2 in a PLC ϵ -dependent manner. Under our experimental conditions, induction of IL-1 β was not detected in fibroblasts (data not shown). In contrast, keratinocytes cultured in the low-calcium media, which were almost negative for keratin 1 (Fig. 7A), failed to show PLC ϵ -dependent induction of any of the cytokines tested on stimulation with IL-17, IFN- γ , TNF- α , and IL-4 (data not shown). However, when keratinocytes were induced to differentiate in the high-calcium media containing 0.5 mM Ca $^{2+}$ and became highly positive for keratin 10 and keratin 1 (Fig. 7A), marked production of IL-1 β was observed on stimulation with the combination of IL-17 and TNF- α or of IL-17 and IL-4 in a PLC ϵ -dependent manner (Fig. 7B, 7C), suggesting that the differentiation status of keratinocytes may affect this induction. Stimulation with the combination of IL-17 and IFN- γ or with IL-17, IFN- γ , TNF- α , or IL-4 alone failed to cause PLC ϵ -dependent induction of cytokines such as IL-1 α in differentiated keratinocytes (Supplemental Fig. 8). These results indicated that PLC ϵ plays a crucial role in induction of proinflammatory cytokines at the challenged sites, where infiltrated CD4 $^{+}$ T cells produce their intrinsic cytokines, thereby augmenting local inflammatory reactions.

Indirect activation of PLC ϵ by T cell-derived cytokines

To gain mechanistic insights into the role of PLC ϵ in cytokine induction, we examined the kinetics of the cytokine expression from cultured skin cells by measuring mRNA levels at 3 and 12 h after

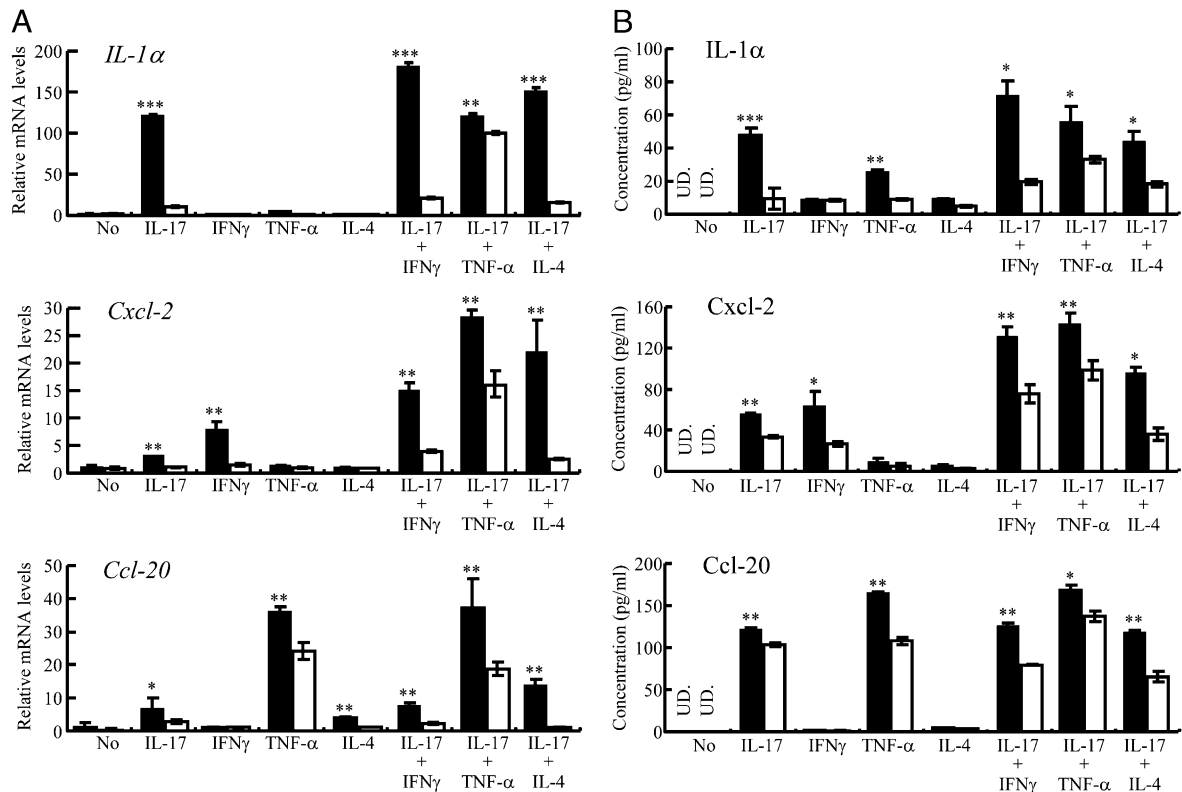


FIGURE 6. Effects of the PLCε background on induction of cytokine expression by T cell-derived cytokines in dermal fibroblasts. Primary cultures of dermal fibroblasts, established from the skins of PLCε^{+/+} (black bars) and PLCε^{-/-} (white bars) mice, were stimulated with the indicated recombinant T cell-derived cytokines at 10 ng/ml for 12 h. The mRNA levels of the indicated cytokines were measured by QRT-PCR followed by normalization to the mRNA level in PLCε^{+/+} mice-derived cells without stimulation (No) to express relative mRNA levels (A). Cytokines secreted into the culture supernatant was determined by ELISA (B). Data are shown as the means ± SD determined from three plates of cells for each PLCε genotype. **p* < 0.05; ***p* < 0.01; ****p* < 0.001 between PLCε^{+/+} and PLCε^{-/-} mice. UD, below detection limit.

stimulation with T cell-derived cytokines, representing the early and middle phases, respectively, of the elicitation stage (Fig. 8A, 8B). As a control, we first tested the induction of IL-1α and CXCL-2 from dermal fibroblasts on stimulation with PMA, which had been shown to occur through activation of PLCε in a manner dependent on both PKC and RasGRP3 (12). The IL-1α level showed robust elevation in a PLCε-dependent manner at 3 h and subsequently returned to almost the basal levels by 12 h. CXCL-2 showed a similar expression pattern although the level and PLCε-dependence of the induction were less apparent. In contrast, CCL-20 failed to show any induction. These data were consistent with those reported previously (12). In contrast, stimulation with IL-17 resulted in modest elevation of IL-1α at 3 h, which was independent of the PLCε background. After that, the IL-1α level exhibited a marked increase at 12 h with clear dependence on the PLCε background. Likewise, CXCL-2 and CCL-20 also showed a PLCε-dependent increase at 12 h after IL-17 administration. Similar time courses were observed with both the TNF-α-stimulated induction of CCL-20 and the IFN-γ-stimulated induction of CXCL-2. In the case of the IL-4-stimulated CCL-20 induction, the CCL-20 level showed increase only at 12 h, which was dependent on the PLCε background. We next carried out similar experiments with differentiated keratinocytes (Fig. 8B). We chose IL-1β as an output because IL-1β, but not IL-1α, was found to be induced in a PLCε-dependent manner (Supplemental Fig. 8). Stimulation with the combination of IL-17 and TNF-α or of IL-17 and IL-4 resulted in robust elevation of IL-1β at 12 h, which showed clear dependence on the PLCε background. No elevation was observed at 3 h. These results demonstrated delayed kinetics of PLCε-dependent cytokine induction by T cell-derived cytokines compared

with that by PMA, hinting that the T cell-derived cytokines may not correspond to the upstream ligand directly coupled to PLCε.

To test this possibility, we examined the activation of PLCε in cultured dermal fibroblasts upon stimulation with PMA or the T cell-derived cytokines by monitoring elevation of the cytosolic-free calcium concentration (Fig. 8C). As reported (12), PMA stimulation induced rapid and sharp elevation of the calcium concentration. In contrast, stimulation with IL-17 failed to induce even slight elevation of the calcium concentration. Essentially, similar results were obtained with stimulation by TNF-α, IL-4, or IFN-γ (data not shown).

Involvement of soluble factors in mediating the T cell cytokine-induced cytokine expression in dermal fibroblasts

The results described previously hinted the existence of an identified factor (factors), which is (are) secreted from skin cells on stimulation by the T cell-derived cytokines and induces (induce) expression of proinflammatory cytokines from skin cells through PLCε activation. To test this possibility, we examined the induction of the IL-1α, CXCL-2, and CCL-20 mRNAs in cultured dermal fibroblasts carrying the different PLCε backgrounds by treatment with the conditioned medium from IL-17-stimulated dermal fibroblasts (Fig. 9). After the addition of the conditioned medium, PLCε-dependent induction of CXCL-2 and CCL-20 was detected at 3 h (Fig. 9), which was much earlier than the time triggered by the direct IL-17 stimulation (Fig. 8A). In contrast, the induction of IL-1α failed to show significant PLCε-dependency at 3 h after the addition of the conditioned medium (Fig. 9). Such difference in the kinetics of cytokine induction suggests that more than one soluble factor

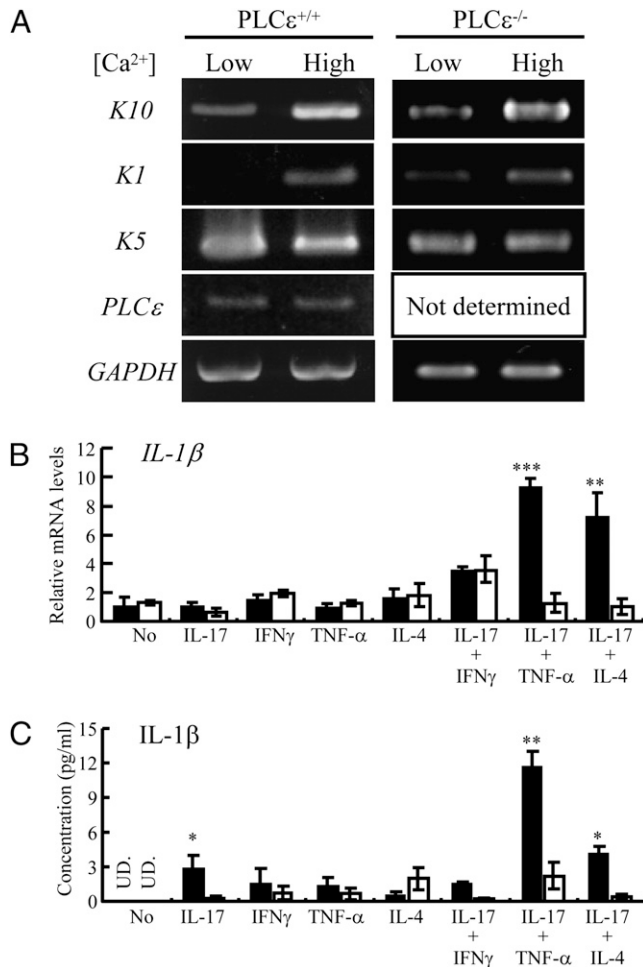


FIGURE 7. Effects of the PLC ϵ background on induction of cytokine expression by T cell-derived cytokines in keratinocytes. **A**, Assessment of keratinocyte differentiation. Epidermal keratinocytes, prepared from the skins of mice with the indicated PLC ϵ genotype, were cultured under low-calcium or high-calcium condition for 2 d and examined for expression of various keratins (K), PLC ϵ , and GAPDH by RT-PCR. **B** and **C**, Measurement of cytokine expression. Expression of cytokines in primary cultures of PLC $\epsilon^{+/+}$ (black bars) or PLC $\epsilon^{-/-}$ (white bars) keratinocytes after stimulation with the indicated recombinant T cell-derived cytokines were assessed by QRT-PCR (**B**) and ELISA (**C**) as described in Fig. 6. * p < 0.05; ** p < 0.01; *** p < 0.001 between PLC $\epsilon^{+/+}$ and PLC $\epsilon^{-/-}$ cells. UD, below detection limit.

secreted from IL-17-activated cells is involved in the cytokine gene activation via receptor engagement (Fig. 10).

Discussion

In the current study, we have shown that PLC ϵ plays a crucial role in induction of the inflammatory reactions in the elicitation stage of CHS. Our observation that the extents and kinetics of CD4⁺ T cell infiltration were not affected by the PLC ϵ background (Fig. 5, A–C) and that PLC ϵ is not expressed in CD4⁺ T cells (Fig. 4) indicated that PLC ϵ exerts its function in cells other than CD4⁺ T cells, presumably, fibroblasts or keratinocytes of the skin, to augment inflammatory reactions. This was supported by the results of the adoptive transfer experiments of CD4⁺ T cells from DNFB-sensitized animals (Fig. 5D, 5E). Expression of proinflammatory cytokines, such as IL-1 α , IL-1 β , CXCL-1, CXCL-2, and CCL-20, in both epidermal keratinocytes and dermal fibroblasts was markedly attenuated in PLC $\epsilon^{-/-}$ mice as assessed by QRT-PCR and immunostaining (Figs. 2, 3). Given that these cytokines have

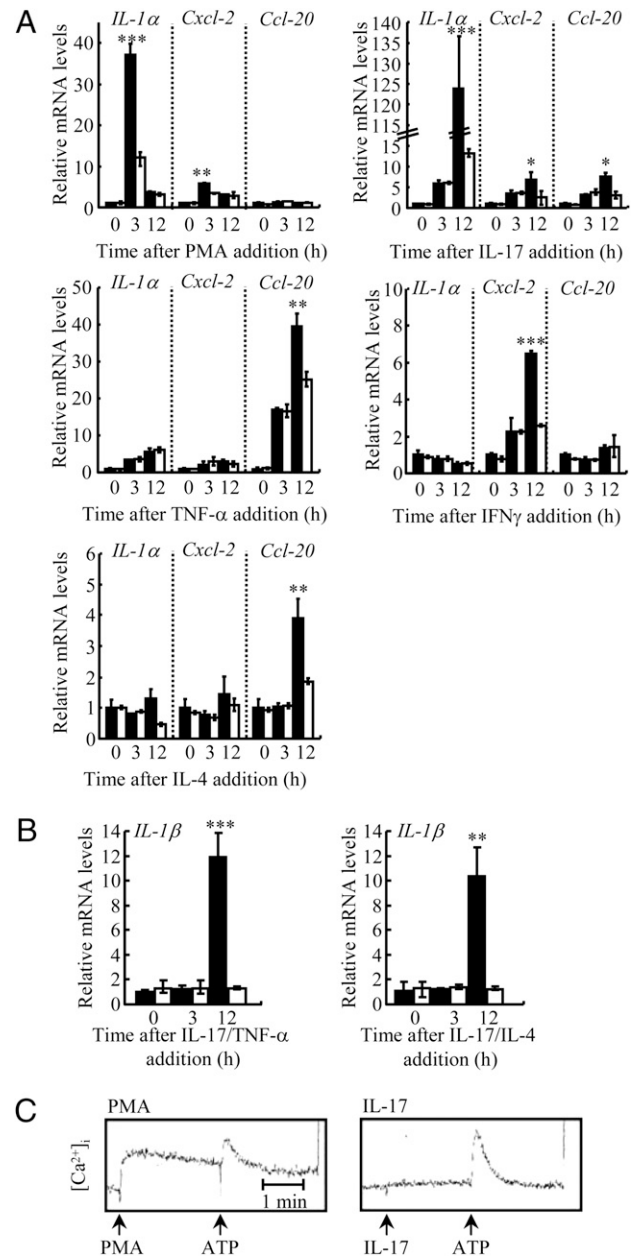


FIGURE 8. Analysis of the role of PLC ϵ in signaling downstream of T cell-derived cytokines. **A** and **B**, Kinetics of the induction of cytokine mRNA expression. Primary cultures of dermal fibroblasts (**A**) and epidermal keratinocytes (**B**), established from the skins of PLC $\epsilon^{+/+}$ (black bars) and PLC $\epsilon^{-/-}$ (white bars) mice, were stimulated with the indicated recombinant T cell-derived cytokines (10 ng/ml) or PMA (10 ng/ml) for the indicated periods, and the mRNA levels of the indicated cytokines were measured by QRT-PCR. Relative mRNA levels normalized to the mRNA level in PLC $\epsilon^{+/+}$ mice-derived cells at 0 h are shown as the means $\pm$ SD of triplicate determinations. ** p < 0.01; *** p < 0.001 between PLC $\epsilon^{+/+}$ and PLC $\epsilon^{-/-}$ mice. **C**, Effect of IL-17 stimulation on activation of PLC ϵ . Fura-2-loaded dermal fibroblasts were stimulated with IL-17 (10 ng/ml), PMA (10 ng/ml), or ATP (250 μ M) at the indicated time points. PLC ϵ activation was determined as the increase of the cytosolic-free calcium concentration. Relative increases in the calcium concentrations were calculated as described in *Materials and Methods*.

pleiotropic functions such as upregulation of the expression of cell adhesion molecules (27) and chemoattracting activity toward granulocytes and immature dendritic cells (28–30), the PLC ϵ 's function in augmenting inflammatory reactions is possibly mediated by the induction of their expression. In addition, experiments

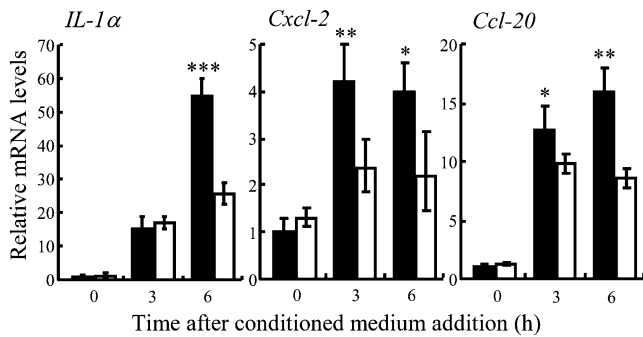


FIGURE 9. Analysis of the cytokine-inducing activity of the conditioned medium from IL-17-stimulated dermal fibroblasts. The conditioned medium from primary-cultured PLCε^{+/+} dermal fibroblasts stimulated with IL-17 was added to PLCε^{+/+} (black bars) and PLCε^{-/-} (white bars) fibroblasts, and the mRNA levels of the indicated cytokine genes were assessed by QRT-PCR as described in Fig. 6. **p* < 0.05; ***p* < 0.01; ****p* < 0.001 between PLCε^{+/+} and PLCε^{-/-} mice.

with primary cultures of epidermal keratinocytes and dermal fibroblasts showed that T cell-derived cytokines, such as IL-17A, IFN-γ, TNF-α, and IL-4, alone or in combination, were capable of inducing expression of proinflammatory cytokines, such as IL-1α, IL-1β, CXCL-2, and CCL-20, in a PLCε-dependent manner (Figs. 6, 7). These results taken together imply that PLCε plays a crucial role in induction of proinflammatory cytokines from keratinocytes and fibroblasts at the challenged sites, where infiltrated CD4⁺ T cells produce their intrinsic cytokines, thereby augmenting local inflammatory reactions. Such a function of PLCε in inflammation is unique among the PLC isozymes because PLC isozymes of other classes were reported to contribute to inflammation through distinct ways; PLCγ2, which is most highly expressed in immune cells, regulates activation of immune cells, thereby inducing immune inflammatory reactions (31, 32) and PLCδ1 negatively regulates expression of proinflammatory cytokines such as IL-1β in keratinocytes (31, 33).

As for the mechanism underlying the PLCε's action, the delayed kinetics of the proinflammatory cytokine induction by the T cell-derived cytokines (Fig. 8A, 8B), the failure of the T cell-derived cytokines in inducing PLCε activation in dermal fibroblasts (Fig. 8C), and difference in the kinetics of the cytokine induction in cells treated with the conditioned medium from IL-17-stimulated cells (Fig. 9) suggested that the cytokine-inducing function of the T cell-derived cytokines is mediated by soluble factors, which are produced by fibroblasts or/and keratinocytes in a PLCε-independent manner on stimulation with the T cell-derived cytokines (Fig. 10). An example of delayed activation of an intracellular signaling pathway probably mediated by the production of secondary factors was reported in the case of IL-17-stimulated ERK activation in mouse embryonic fibroblasts mediated by Ras and/or Rap signaling, where ERK was markedly activated by 24 h IL-17 treatment but not by short-term (~10–30 min) treatment (34). Further studies including the identification of such factor(s) will be needed to clarify the whole picture of the signaling pathway(s) mediated by PLCε leading to cytokine gene activation.

It should be noted that some discrepancies exist between the expression patterns of the proinflammatory cytokines observed in vivo and in vitro. Although fibroblasts and keratinocytes in culture showed PLCε-dependent expression of different sets of the cytokines, IL-1α, CXCL-2, and CCL-20, versus IL-1β, on stimulation with the T cell-derived cytokines (Figs. 6–8), these cells in vivo almost equally produced all the four cytokines in a PLCε-dependent manner (Fig. 3). Also, the differentiation status-dependent expression of IL-1β in

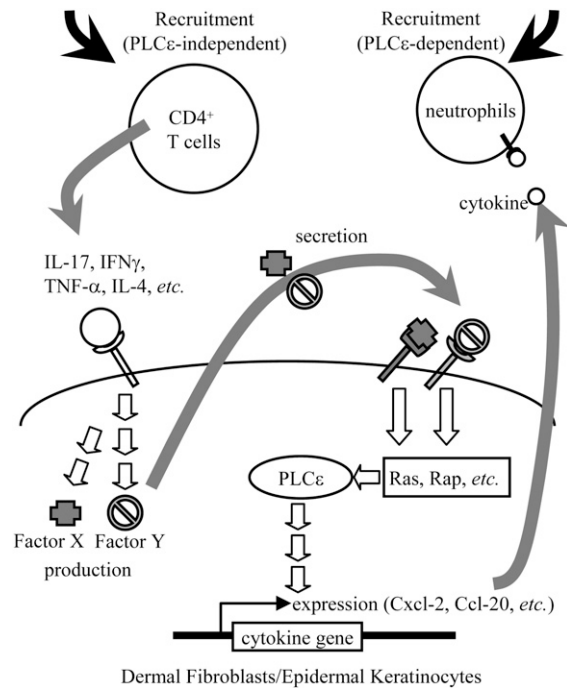


FIGURE 10. Model of the development of inflammatory reactions in the elicitation stage of CHS. On a DNFB challenge, CD4⁺ T cells infiltrate to the challenged sites and secrete cytokines, such as IL-17, IFN-γ, TNF-α, and IL-4, which subsequently bind to their receptors on fibroblasts and keratinocytes, leading to production and secretion of more than one unidentified soluble factor (represented by factors X and Y) in a PLCε-independent manner. Such soluble factors are likely to be linked to the activation of PLCε, leading to the expression of proinflammatory cytokines including CXCL-2 and CCL-20. These cytokines induce neutrophil recruitment etc., leading to full-blown inflammation.

cultured keratinocytes (Fig. 7) was apparently not observed in vivo (Fig. 3). These discrepancies could be explained by the involvement of other intercellular molecules not examined in the current study, which are produced from skin constituents excluding T cells and stimulate fibroblasts or keratinocytes to induce PLCε-dependent expression of certain cytokines missing in the in vitro study.

It is of particular interest that IL-17 is capable of inducing the cytokine expression in a PLCε-dependent manner because IL-17 is a pleiotropic proinflammatory cytokine having a wide array of target cells (3, 35) and implicated in the development of a variety of inflammatory diseases, including not only CHS, but also rheumatoid arthritis (36), psoriasis (37, 38), inflammatory bowel disease (39), and asthma (40). Also, the observation that TNF-α induced the proinflammatory cytokine expression in a PLCε-dependent manner is reminiscent of the crucial roles of PLCε in skin chemical carcinogenesis (11, 41–43) and Apc^{Min/+}-dependent intestinal tumorigenesis (13, 42, 43). The roles of PLCε in various inflammatory diseases are under investigation in our laboratory. The results of these studies as well as the current one may lay the solid foundation in introducing PLCε as a promising molecular target for the development of novel anti-inflammatory drugs.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Figure Legends

Supplemental Figure 1. Identification of neutrophils by two different methods. *A*, Identification by H&E staining. A representative specimen of the inflamed skin of the PLC $\epsilon^{+/+}$ ear at 24 h after DNFB challenge is shown. A right picture is an enlargement of the indicated area of the left picture. *Black arrows* and *asterisks* denote neutrophils and other leukocytes, respectively. *Bar* in the left panel, 100 μ m. *B*, Identification by immunostaining with anti-Gr-1 Ab. Representative specimens of the inflamed skin of the ear at 24 h after DNFB challenge are shown. Nuclei were visualized by DAPI staining. *Bars* on the right margins of the pictures denote the epidermis (*blue*), dermis (*red*) and auricular cartilage (*green*). *Bars*, 200 μ m. *C*, Comparison of the data obtained with two different methods for the identification of the neutrophils. Neutrophils were counted on the skin sections stained by the indicated methods at 24 h after the DNFB challenge. Data are expressed as the means $\pm$ SD *p* values are shown. (*n* = 3 per group)

Supplemental Figure 2. Comparison of DNFB-induced CHS reactions between PLC $\epsilon^{+/+}$ and PLC $\epsilon^{-/-}$ mice. Ears of the sensitized male mice were painted with acetone vehicle (*A* and *B*) or DNFB (*C* and *D*). Twenty-four hours later, the ear auricles of PLC $\epsilon^{+/+}$ (*A* and *C*) and PLC $\epsilon^{-/-}$ (*B* and *D*) mice were collected and subjected to preparation of H&E-stained sections. *Bars*, 200 μ m.

Supplemental Figure 3. Immunostaining analysis of the cytokine production. Cytokines in the ears of PLC $\epsilon^{+/+}$ (+/+) and PLC $\epsilon^{-/-}$ (-/-) mice were detected by immunostaining with their specific Abs (*red*) at 24 h after the challenge with DNFB or with acetone vehicle. *Bars* on the right margins of the pictures denote the epidermis (*blue*), dermis (*red*) and auricular cartilage (*green*). *Bars*, 200 μ m.

Supplemental Figure 4. Demonstration of the absence of PLC ϵ in CD4⁺ T cells. Expression of PLC ϵ (*A*, *green*) and PLC γ 1 (*B*, *green*) in CD4⁺ T cells (*red*) was assessed by immunostaining of the cross sections of the ears of a PLC $\epsilon^{+/+}$ mouse at 24 h after the DNFB challenge. Nuclei were visualized by DAPI staining. A merged image of all the 3 staining and its enlargement are also shown. *Bars*, 200 μ m.

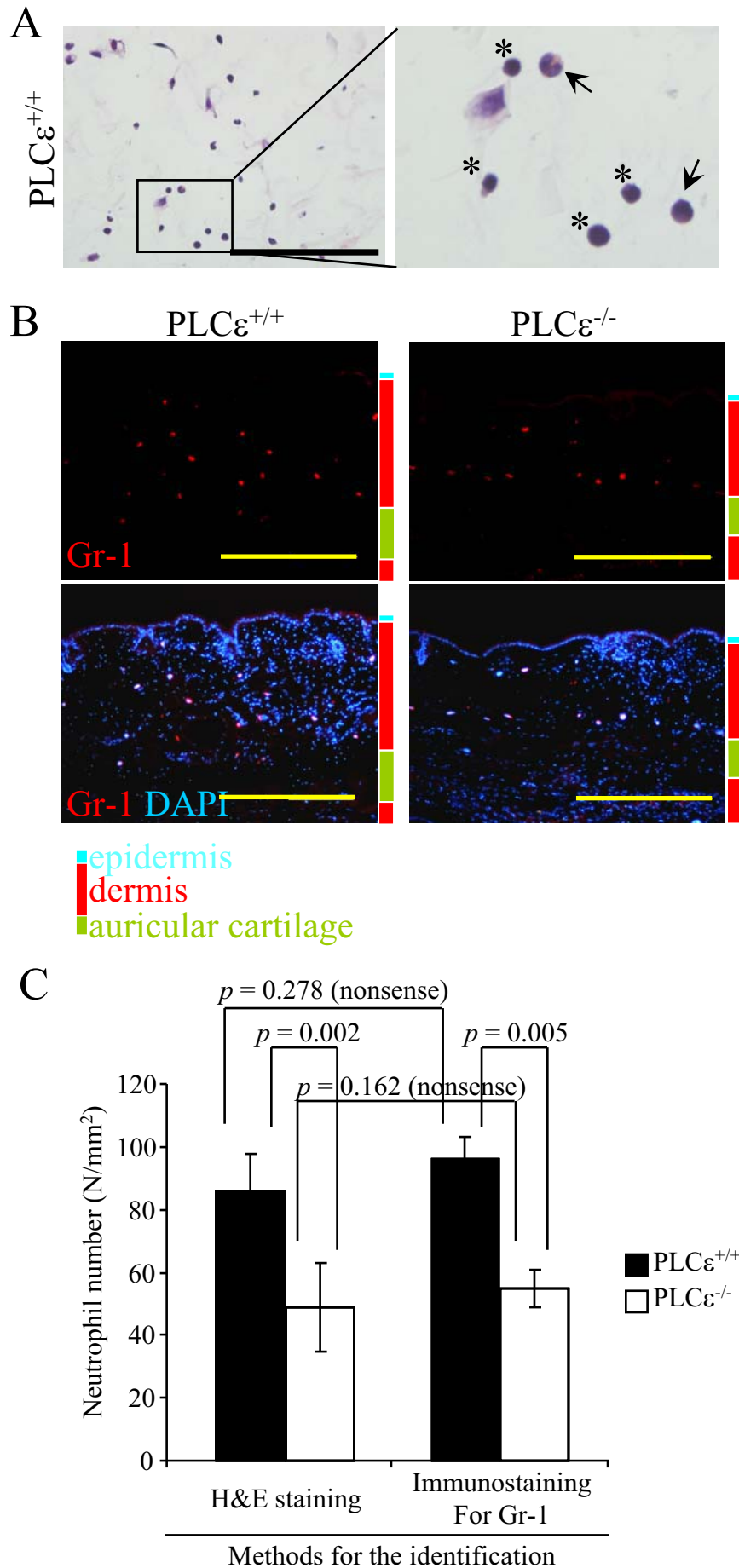
Supplemental Figure 5. Demonstration of the absence of the PLC ϵ in CD4⁺ T cells by RT-PCR. First-strand cDNAs used in Fig. 4D [RT(+)] were subjected to PCR with an excessive number of

amplification cycles (60 cycles) using 2 different primer sets (Set A and Set B as in Fig. 4). In these conditions, non-specific PCR products were generated even by the reactions without reverse-transcriptase [RT(-)], supporting the notion that PLC ϵ is absent in CD4⁺ T cells.

Supplemental Figure 6. qRT-PCR analysis of IL-22. qRT-PCR was performed to determine the levels of the β -actin and IL-22 mRNAs in the ear skin of DNFB-sensitized PLC $\epsilon^{+/+}$ and PLC $\epsilon^{-/-}$ animals at 0, 3, and 24 h after the DNFB challenge. Each line indicates the fluorescence intensity of the SYBR-intercalated PCR product obtained from each cDNA template.

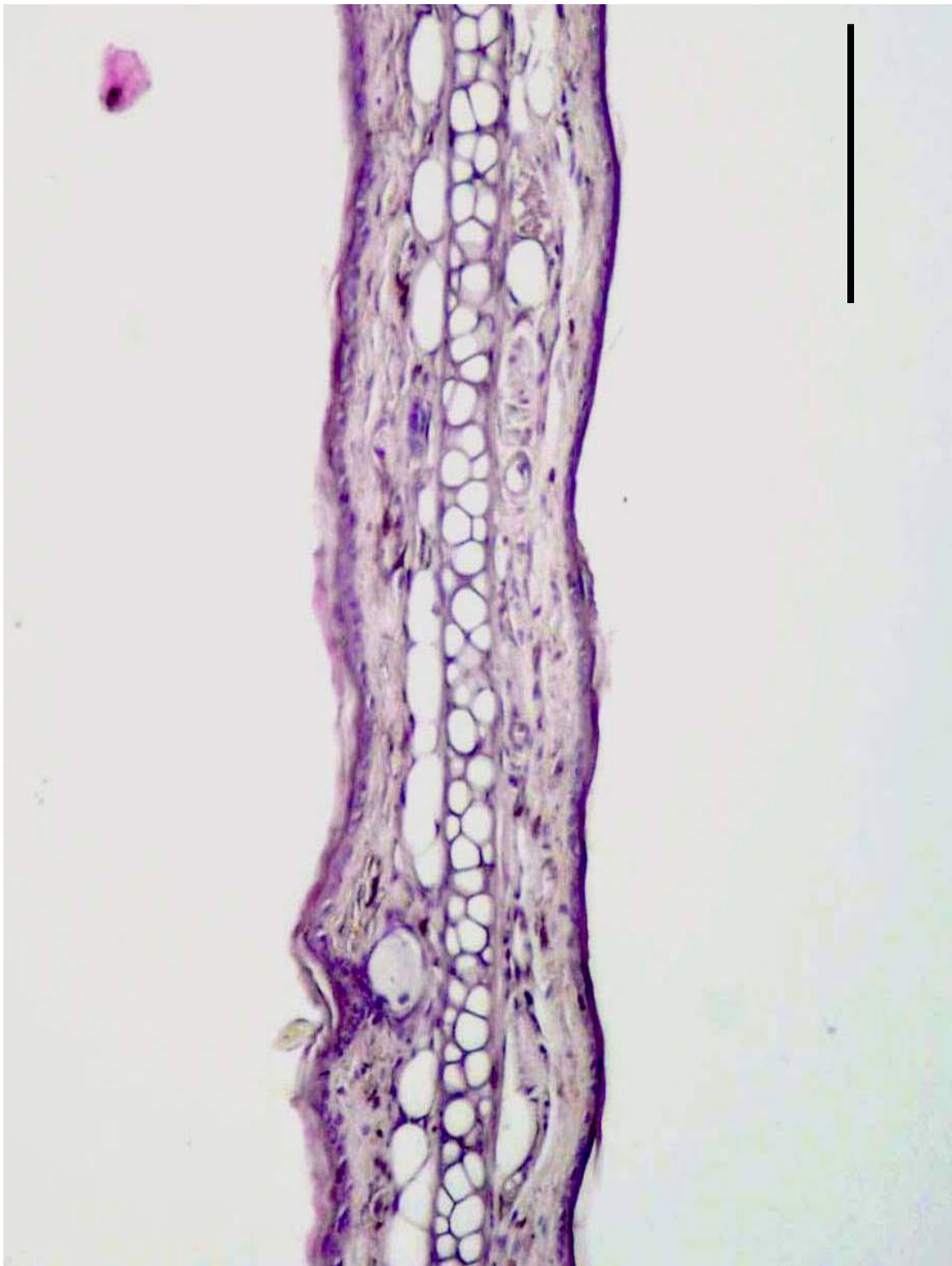
Supplemental Figure 7. Immunostaining analysis of the CD4⁺ T cell infiltration. Cross sections of the ears of PLC $\epsilon^{+/+}$ and PLC $\epsilon^{-/-}$ mice at 24 h after the DNFB challenge were subjected to immunostaining for CD4 (*red*). Nuclei were visualized by DAPI staining (*blue*). Bars, 200 μ m.

Supplemental Figure 8. Effects of the PLC ϵ background on induction of cytokine expression by T cell-derived cytokines in differentiated keratinocytes. After differentiated, primary-cultured keratinocytes were stimulated with the indicated recombinant T cell-derived cytokines for 12 h. Semi-quantitative RT-PCR was performed to detect the cytokines.

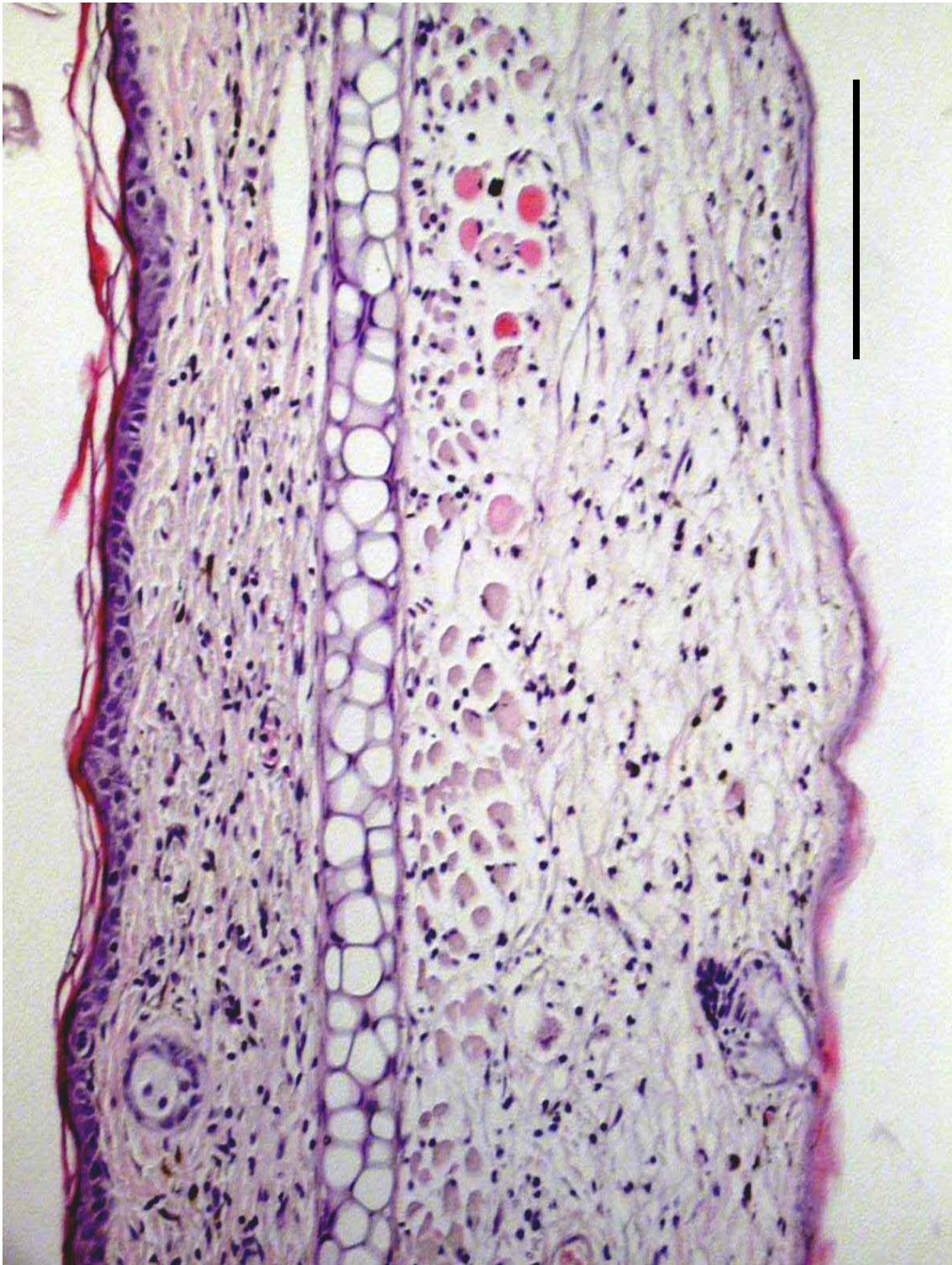




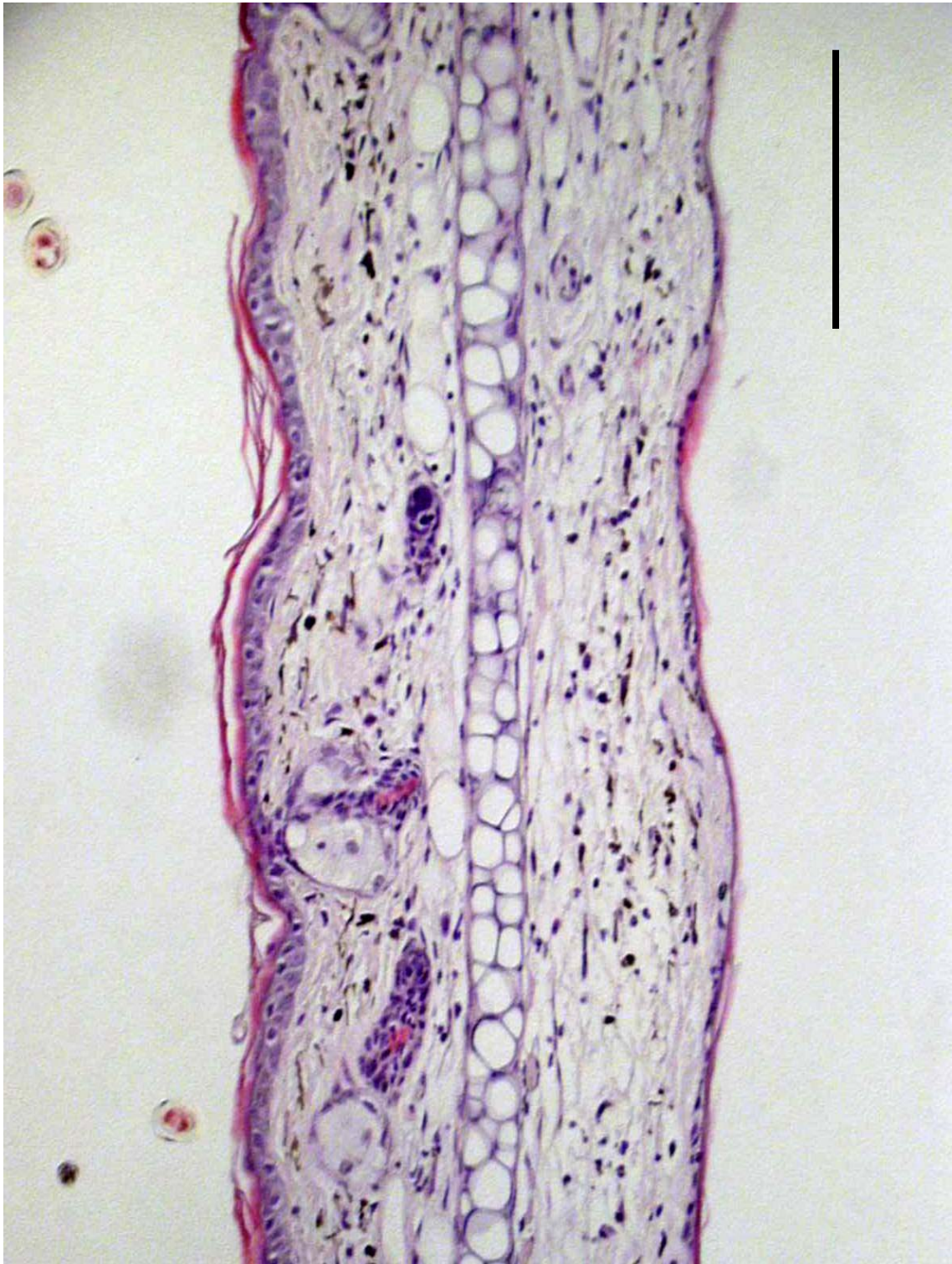
PLC $\epsilon^{+/+}$ (acetone vehicle)



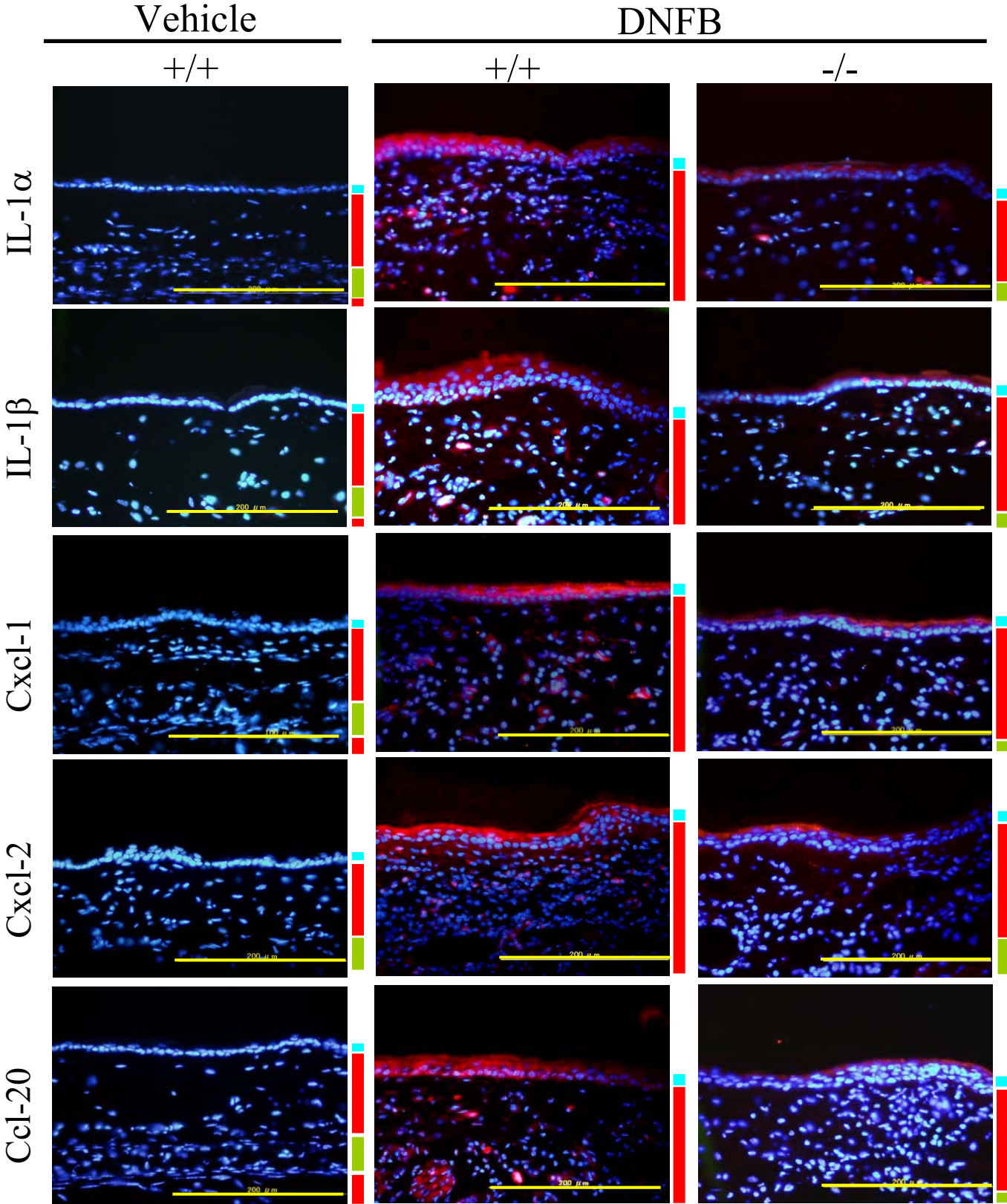
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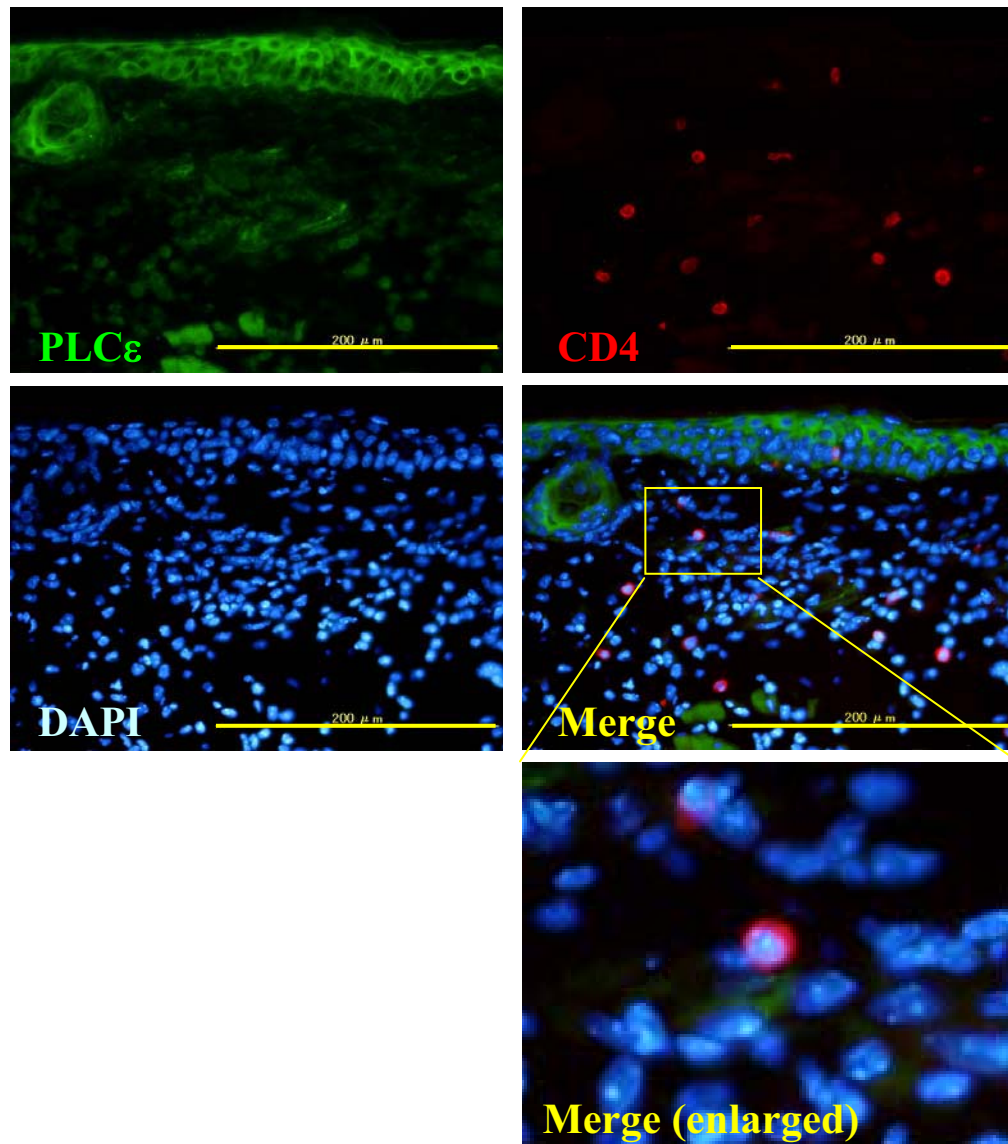
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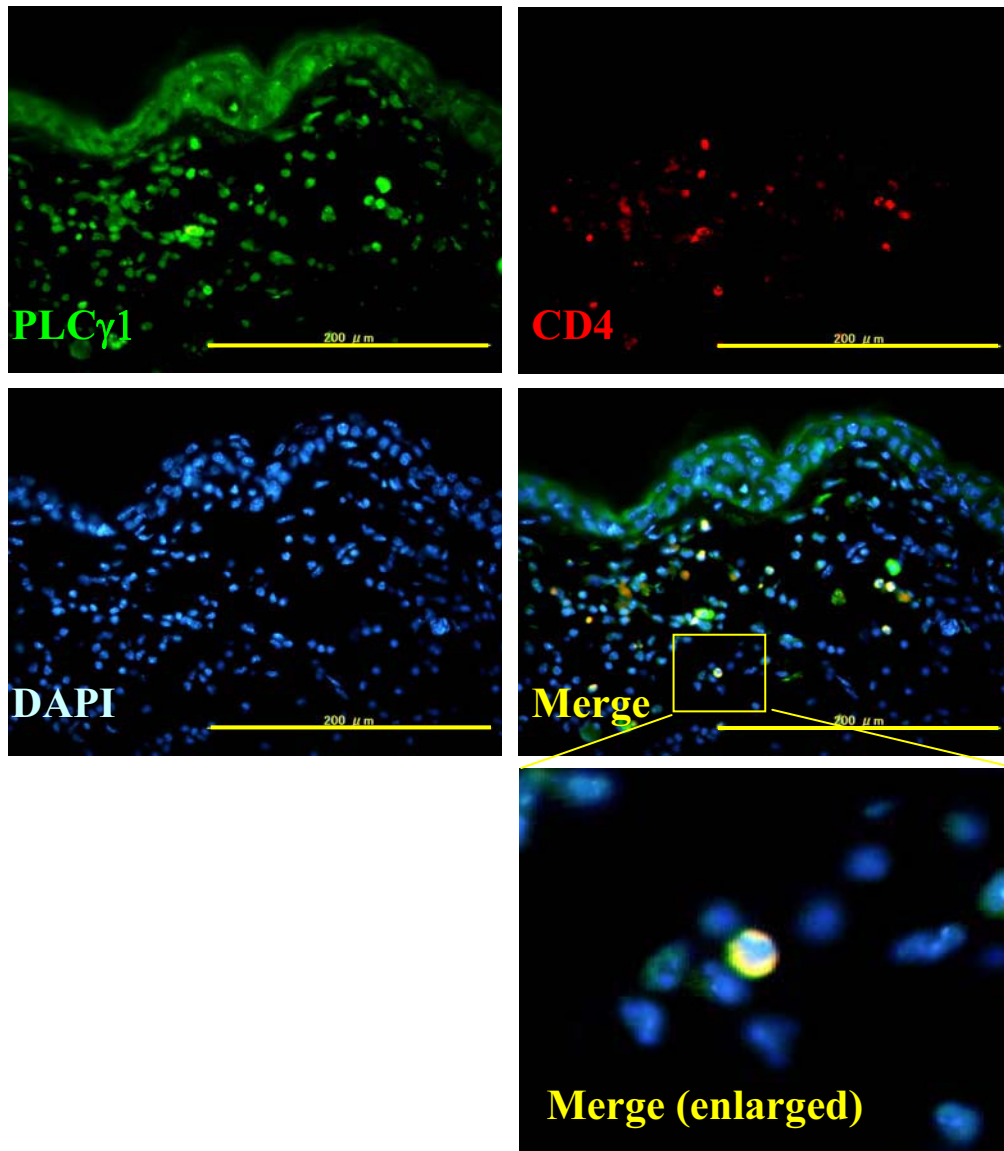


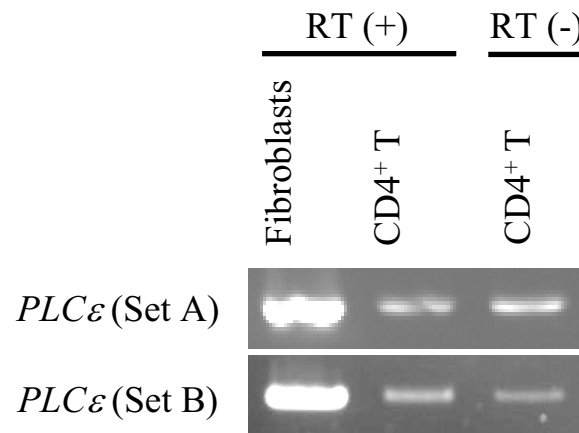
PLC $\epsilon^{-/-}$ (DNFB)

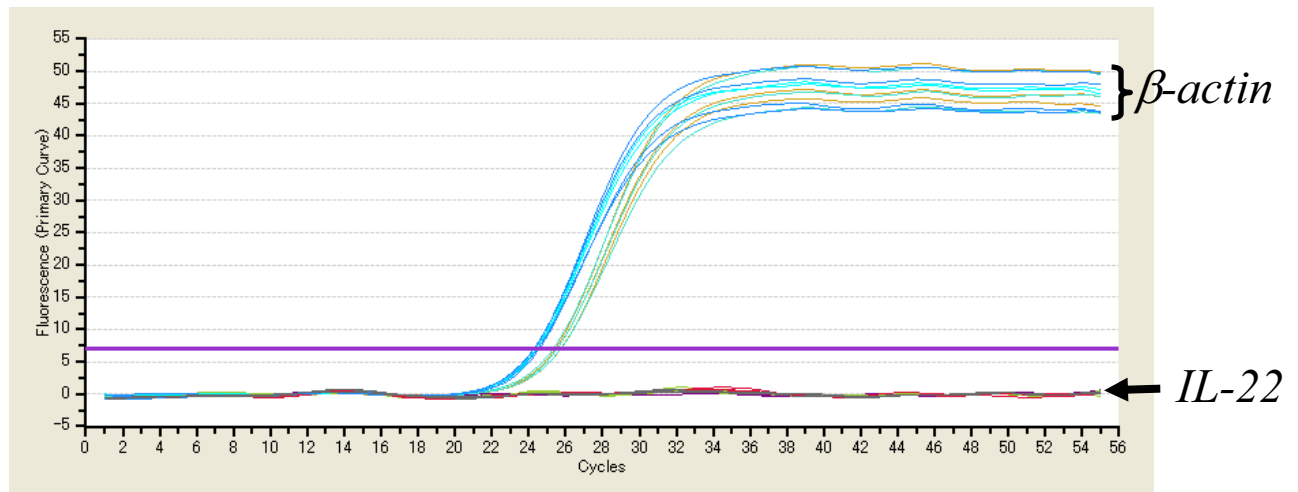


■ epidermis
■ dermis
■ auricular cartilage

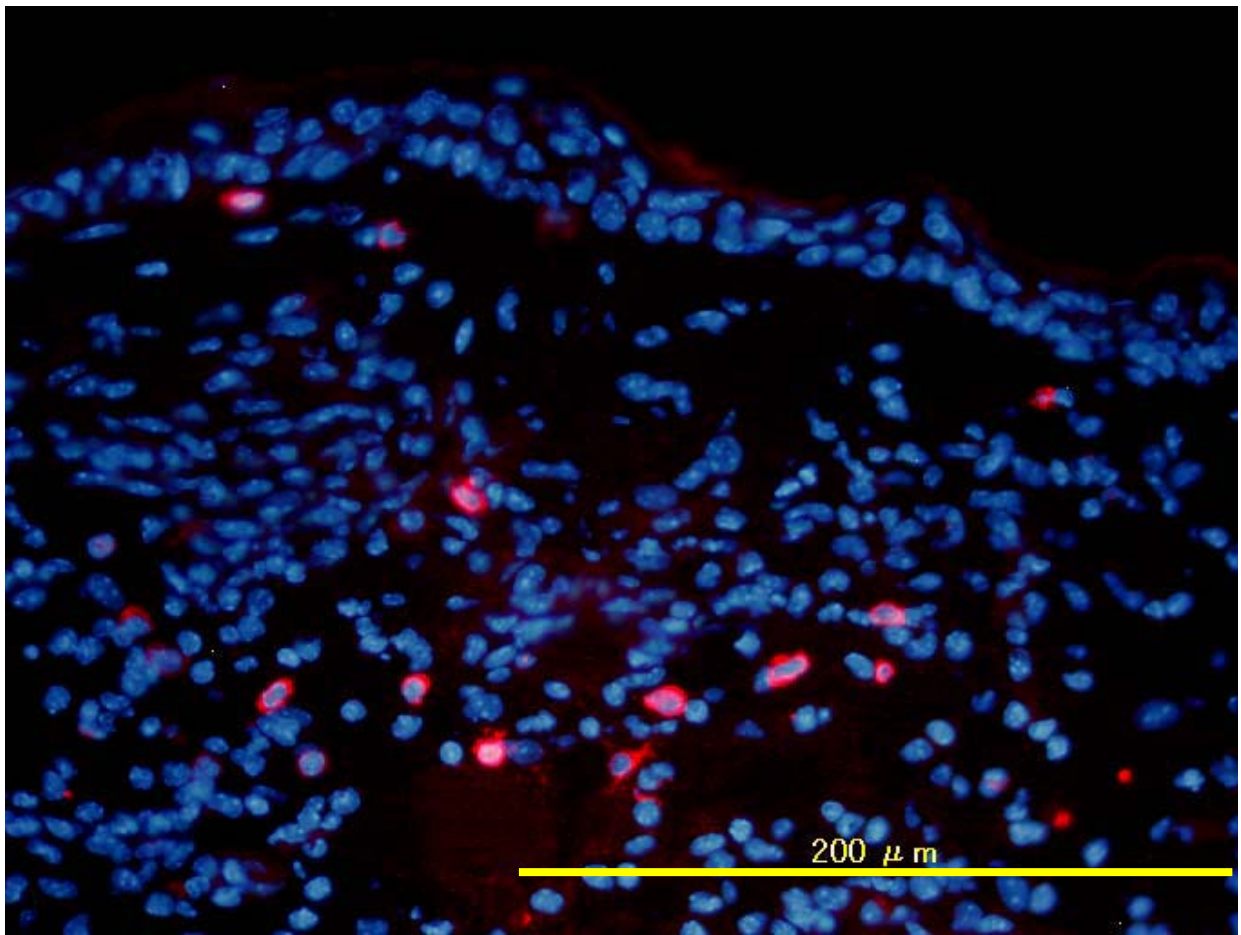








PLC $\epsilon^{+/+}$



PLC $\epsilon^{-/-}$

