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IL-36 α is involved in hapten-specific T-cell induction, but not local inflammation, during contact hypersensitivity

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ABSTRACT

Levels of IL36 α are known to be increased in specimens from patients with atopic dermatitis and psoriasis. In addition, it has been reported that IL-36 α is crucial for development of imiquimod-induced psoriatic dermatitis in mice. On the other hand, the role of IL-36 α in induction of allergic contact dermatitis/contact hypersensitivity (ACD/CHS) is poorly understood. We found that IL-36 α was produced in keratinocytes of mice during imiquimod-induced psoriatic dermatitis, but it was hardly detectable in the skin of mice during either fluorescein isothiocyanate (FITC)- or 1-fluoro-2, 4-dinitrobenzene (DNFB)-induced CHS. Although IL-36 α can enhance activation of dendritic cells (DCs) and T cells, in CHS, IL-36 α was not essential for DC migration from the skin to draining LNs, but it was required for induction or activation of hapten-specific T cells such as Th/Tc1 or Th17 cells. However, local inflammation, assessed by measurement of ear skin thickness, was comparable between wild-type and IL-36 α -deficient mice during both FITC- and DNFB-induced CHS. These observations indicate that IL-36 α is involved in induction and/or activation of hapten-specific T-cell subsets in the sensitization phase of CHS, but not essential for induction of local inflammation in the elicitation phase.

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1. Introduction

Allergic contact dermatitis/contact hypersensitivity (ACD/CHS), which develops in response to repeated epicutaneous exposure to a hapten [1], is a hapten-specific T-cell-mediated disease. Haptens, which are generally non-immunogenic low-molecular-weight chemicals, are capable of crossing the stratum corneum barrier and forming chemically-modified immunogenic neo-antigens by binding with self-proteins [2]. In the sensitization phase of CHS, haptenized self-proteins are captured by cutaneous dendritic cells (DCs), which then migrate to draining lymph nodes (LNs) and present the haptenized self-proteins to naïve T cells [3,4]. The naïve

T cells subsequently differentiate into hapten-specific effector and memory T cells. In the elicitation phase, hapten-specific T cells in LNs are activated by DCs when re-exposed to the same haptenized self-proteins and then migrate from the LNs to the skin, resulting in induction of local inflammation.

IL-36, which is a member of the IL-1 family of cytokines, consists of three agonists, i.e., IL-36 α , IL-36 β and IL-36 γ (formerly known as IL-1F6, IL-1F8 and IL-1F9, respectively), and an antagonist, i.e., IL-36 receptor antagonist (IL-36RN or IL-36Ra, formerly known as IL-1F5). Those four ligands are produced from distinct genes and bind to the same receptors, consisting of IL-1 receptor like 2 (IL-1RL2; also called IL-1Rrp2) and IL-1R accessory protein (IL-1RAcP; also called IL-1RAP) [5]. IL-36 is expressed in various tissues such as the skin, LNs, tonsils, bone-marrow and/or spleen, in humans and/or mice [5,6]. In addition, IL-36 is constitutively expressed and/or induced in response to certain stimuli in human and/or mouse keratinocytes, epithelial cells, endothelial cells, monocytes/macrophages, DCs and/or T cells [7–9]. IL-1RL2 is expressed in various

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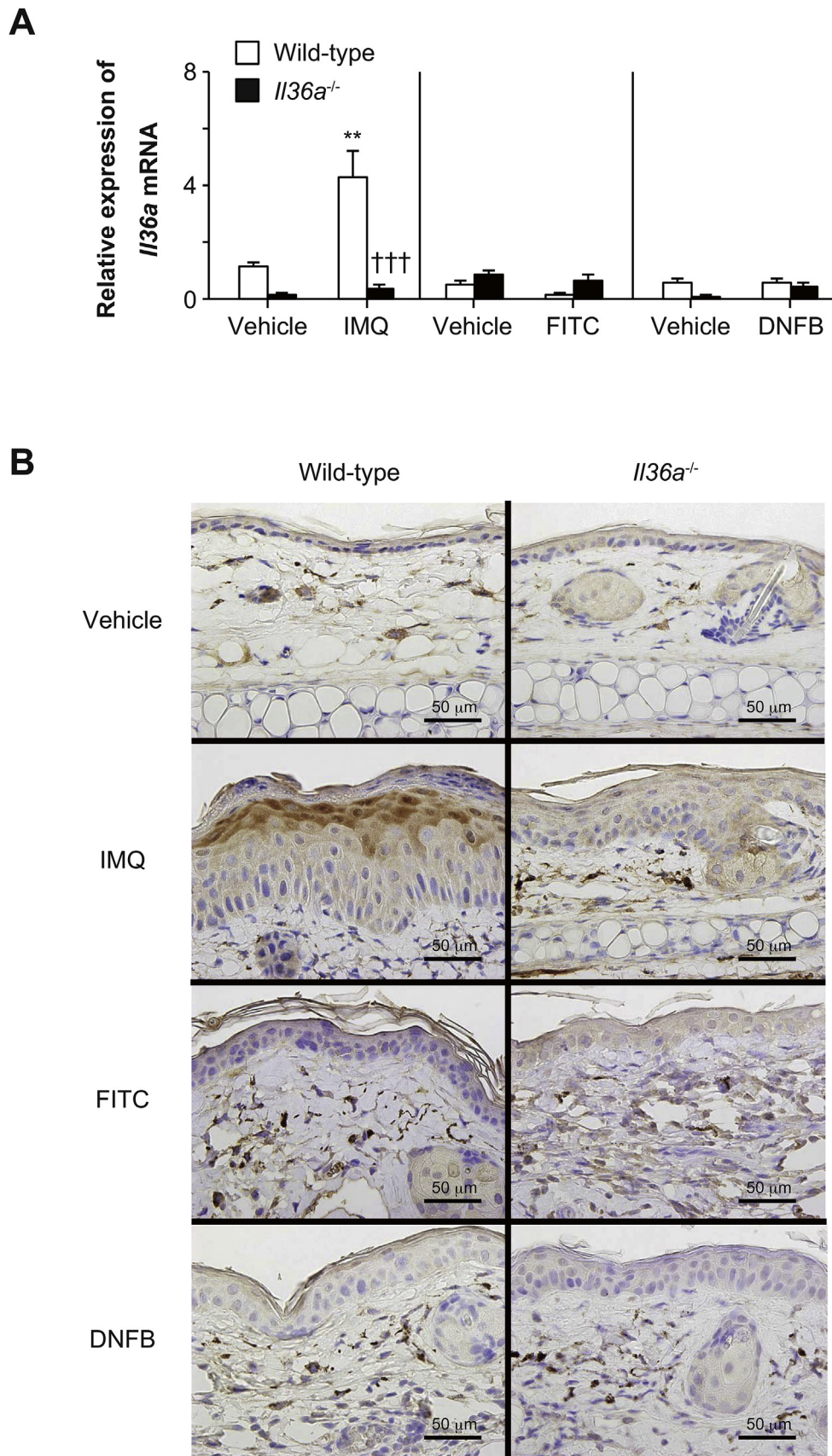


Fig. 1. IL-36 α is induced in keratinocytes in IMQ-induced dermatitis, but not in CHS. (A) qPCR-determined expression levels of *IL36 α* mRNA in vehicle-, IMQ-, FITC- or DNFB-treated ear skin of wild-type mice ($n = 4-6$) and *IL36 α* ^{-/-} mice ($n = 4-6$). Data show the mean $\pm$ SEM, and a representative result from two independent experiments. ** $p < 0.01$, IMQ-treated ear skin vs. vehicle-treated ear skin; +++ $p < 0.001$, wild-type mice vs. *IL36 α* ^{-/-} mice. (B) Immunohistochemical analysis of IL-36 α -positive cells in vehicle-, IMQ-, FITC- or DNFB-treated ear skin.

tissues such as the skin, trachea, kidney, liver and thyroid, and in various types of cells such as CD4⁺ T cells, macrophages, dendritic cells (DCs), neutrophils and keratinocytes [8,10]. It has been shown that IL-36 can enhance T-cell proliferation, promote Th1 and Th17 immune responses [8,11,12] and induce IFN- γ production in combination with IL-2 and IL-12 on CD8⁺ T cells [13]. On the other hand, excessive/inappropriate production of IL-36 may contribute to development of certain chronic inflammatory diseases such as rheumatoid arthritis [14], Sjögren's syndrome [15] and inflammatory bowel diseases [16].

In regard to skin diseases in humans, expression of IL-36 is increased in specimens from patients with ACD/CHS [17,18], atopic dermatitis (AD) [19] and psoriasis [20–22], suggesting that these cytokines may be involved in development of such skin diseases. Indeed, it has been reported that development of imiquimod-induced dermatitis, which is considered to be a model of psoriasis, was impaired in IL-36 α -deficient (*Il36a*^{−/−}) and *Il1rl2*^{−/−} mice, but not *Il36b*^{−/−} or *Il36g*^{−/−} mice [23–25], indicating that IL-36 α , but not IL-36 β or IL-36 γ , is crucial for that development. On the other hand, little is understood regarding whether IL-36 is involved in the pathogenesis of ACD/CHS. Here, we investigated that issue using *Il36a*^{−/−} mice and found that IL-36 α is required for induction of hapten-specific T cells, but not induction of the local inflammation of ACD/CHS in mice.

2. Materials and methods

2.1. Mice

C57BL/6J-wild-type mice were obtained from Sankyo Labo Service Corporation (Tsukuba, Japan). C57BL/6J-*Il36a*^{−/−} mice were

generated as described previously [25]. All mice were housed in a specific-pathogen-free environment at The Institute of Medical Science, The University of Tokyo. The protocol for the animal experiments was approved by the Institutional Review Board of the Institute (A14-10), and all experiments were conducted according to the ethical and safety guidelines of the Institute.

2.2. Induction of dermatitis

Imiquimod (IMQ)-induced dermatitis, and fluorescein isothiocyanate (FITC) and 1-fluoro-2, 4-dinitrobenzene (DNFB) in mice was performed as described elsewhere [24–26].

2.3. Histological analysis

Twenty-four hours after the last treatment with IMQ, FITC or DNFB, the ear skins of mice were harvested, fixed in formalin and embedded in paraffin. Sections were prepared and stained with hematoxylin-eosin. For detection of IL-36 α , sections were incubated in an antigen retrieval solution (HistoVT One; Nacalai Tesque, Inc., Kyoto, Japan) at 95 °C for 40 min and blocked with Blocking One Histo (Nacalai Tesque, Inc.) at room temperature (RT) for 10 min, followed by incubation with a primary Ab (goat anti-mouse IL1f6 mAb; G-13; Santa Cruz Biotechnology, Dallas, TX) at 4 °C overnight. After washing, the samples were incubated with a secondary Ab (rabbit anti-goat Ig Ab; Histofine® Simple Stain™ Mouse MAX PO (G); Nichirei Biosciences Inc., Tokyo, Japan) at RT for 30 min, and developed using ImmPACT™ DAB (SK-4105; Vector Laboratories, Burlingame, CA). Then the sections were counterstained with hematoxylin.

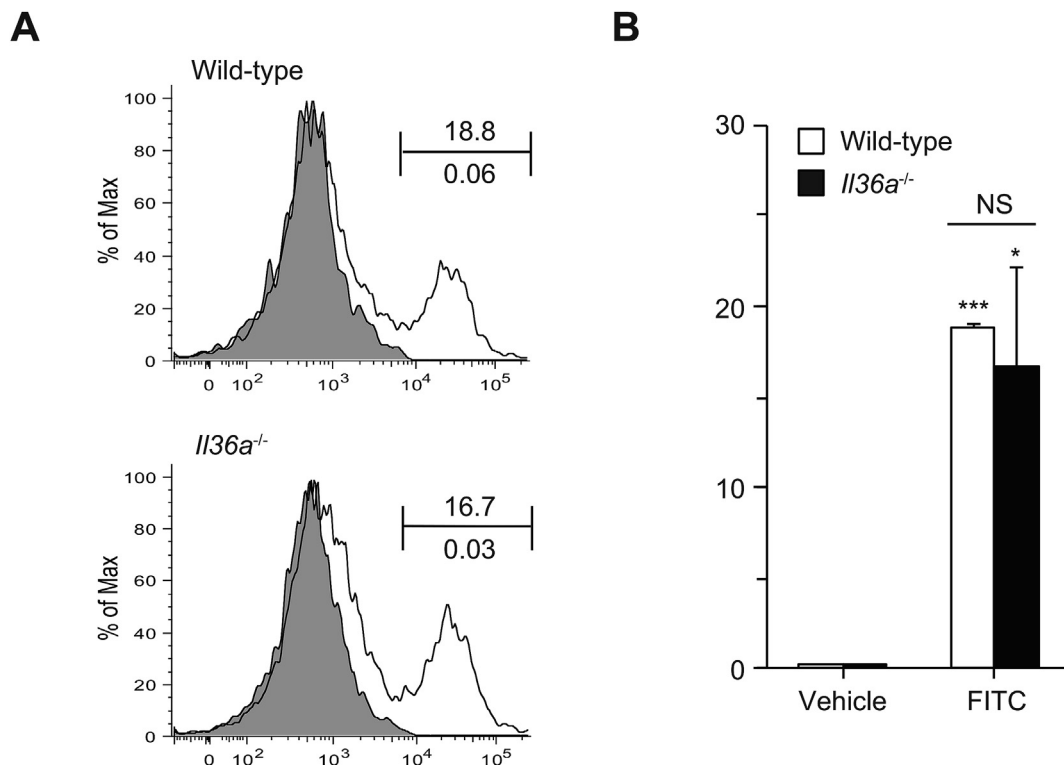


Fig. 2. IL-36 α is not required for migration of DCs from the skin to draining LNs. Flow cytometry was performed to determine the proportion of FITC-positive cells among 7-AAD-negative MHCII^{hi} CD11c⁺ cells in draining LNs from wild-type mice (n = 5) and *Il36a*^{−/−} mice (n = 5) 24 h after epicutaneous treatment with FITC or vehicle alone. (A) Representative flow cytometric data. Shaded area = LNs obtained from vehicle-treated ear skin (lower number [%]); and solid lines = LNs obtained from FITC-treated ear skin (upper number [%]). (B) Data show the mean $\pm$ SEM, and a representative result from two independent experiments. * p < 0.05, and *** p < 0.001, LNs obtained from FITC-treated ear skin vs. LNs obtained from vehicle-treated ear skin. NS, not significant.

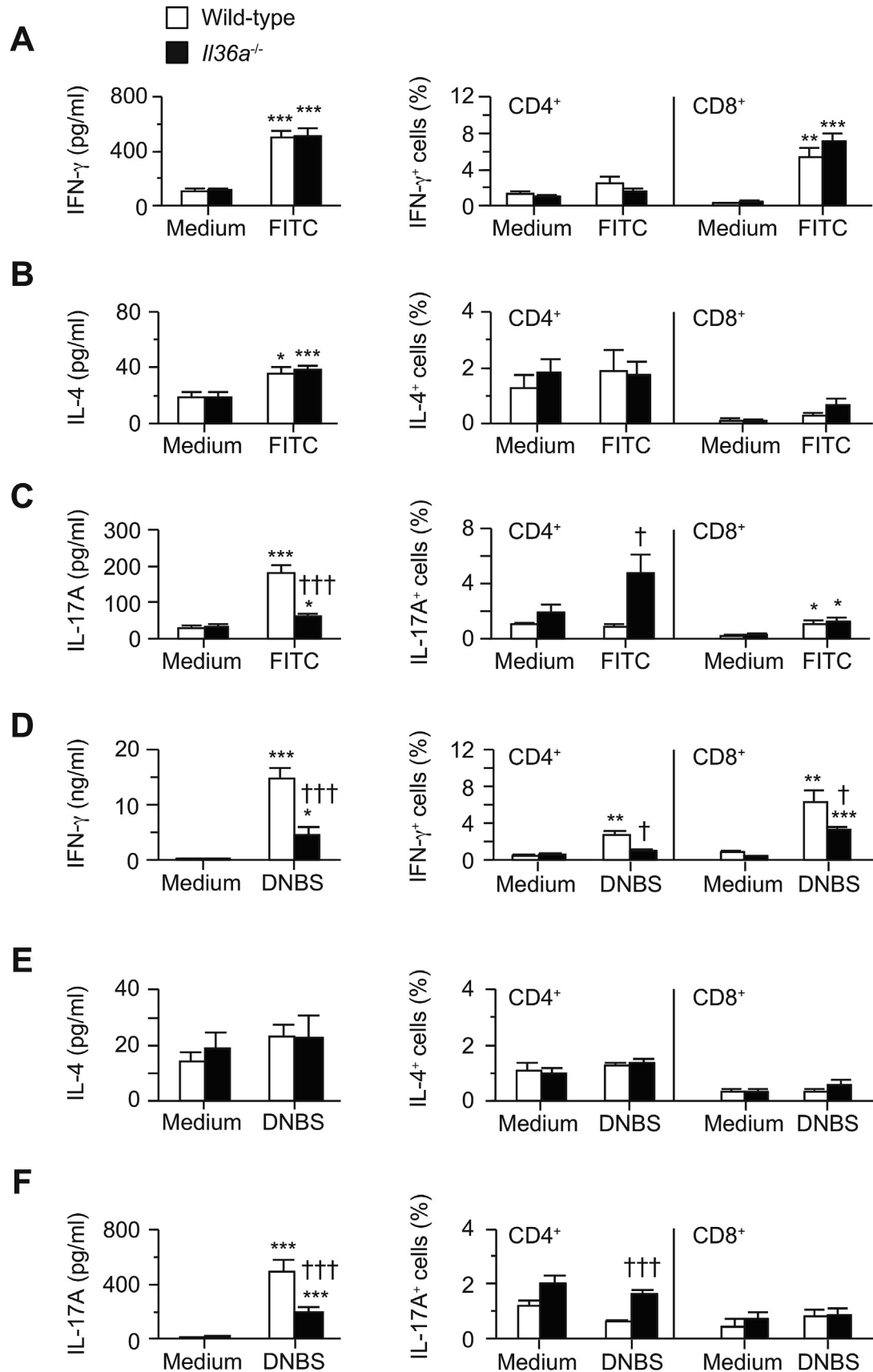


Fig. 3. IL-36 α is involved in hapten-specific T-cell induction. Draining LN cells from wild-type ($n = 5$) and *Il36α*^{-/-} mice ($n = 5$) that had been sensitized with FITC or DNFB were cultured in the presence and absence of 40 μ g/ml FITC or in the presence and absence of 0.5 mM DNBS for 72 h. The levels of IFN- γ , IL-4 and IL-17A in the culture supernatants, and the proportions of IFN- γ ⁺, IL-4⁺ and IL-17A⁺ cells in CD3⁺CD4⁺ cells and CD3⁺CD8⁺ cells in the culture, were determined by ELISA and flow cytometry, respectively. (A–C)

2.4. Quantitative PCR

Total RNA was isolated, and the mRNA expression levels were determined by qPCR as described elsewhere [27]. Relative gene expression was determined against *Gapdh* gene expression. The following PCR primers were designed: forward primer 5'-gcctgtgctacagcttgggg-3' and reverse primer 5'-accagggaaggctcagact-3' for *Il36a* mRNA; and forward primer 5'-cccactcttcaccttcgatg-3' and reverse primer 5'-agggtccaccacctgtgtgct-3' for *Gapdh* mRNA.

2.5. Skin DC migration

Skin DC migration was determined as described elsewhere [26,27]. The percentage of FITC-positive cells among 7-aminoactinomycin D (A9400; 7-AAD; Sigma-Aldrich Co. LLC, St. Louis, MO) -negative MHC Class II^{hi} CD11c⁺ cells was determined using a MACSQuant Analyser (Miltenyi Biotec, Bergisch Gladbach, Germany) with MACSQuantify Software (Miltenyi Biotec) and FlowJo software (Tree Star Inc., Ashland, OR).

2.6. LN cell culture

Hapten-specific LN cell responses were determined as described elsewhere [26,27]. LN cells were cultured in the presence and absence of 40 µg/ml FITC or 0.5 mM sodium 2,4-dinitrobenzenesulfonate hydrate (DNBS; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) at 37 °C for 72 h. Then the culture supernatants were collected for ELISA. For intracellular cytokine staining, LN cells were re-stimulated with 1 µg/ml ionomycin (I0634; Sigma-Aldrich Co. LLC), 100 ng/ml phorbol 12-myristate 13-acetate (PMA; P8139; Sigma-Aldrich Co. LLC) and 1 µM monensin (M5273; Sigma-Aldrich Co. LLC) for 3 h.

2.7. Measurement of cytokines by ELISA

The levels of cytokines in culture supernatants were determined using mouse IFN-γ, IL-4 and IL-17A ELISA kits (Biolegend or BD Biosciences, San Jose, CA).

2.8. Detection of cytokines by flow cytometry

LN cells were cultured as above, incubated with anti-mouse CD16/CD32 mAb for 20 min on ice, and stained with Brilliant Violet 421-conjugated anti-mouse CD4 mAb (GK1.5; BioLegend), V500-conjugated anti-mouse CD3ε mAb (145-2C11; BioLegend) and APC-Cy7-conjugated anti-mouse CD8 Ab (53–6.7; BioLegend) for 40 min on ice. Then the cells were fixed in BD Phosflow™ Fix Buffer I (BD Biosciences) at room temperature for 20 min and permeabilized with a permeabilization buffer (FACS buffer containing 0.1% saponin). After washing, the cells were incubated with PE-conjugated anti-mouse IL-17A mAb (eBio17B7; eBioscience, San Diego, CA), PE-Cy7-conjugated anti-mouse IL-4 mAb (11B11; BioLegend), and APC-conjugated anti-mouse IFN-γ mAb (XMG1.2; BioLegend) in permeabilization buffer at 4 °C for 30 min. The expression profiles of intracellular cytokines in CD3⁺CD4⁺ and CD3⁺CD8⁺ cells were analyzed by flow cytometry, as described above.

2.9. Statistical analysis

A two-tailed unpaired *t*-test was performed to determine statistical significance using Graphpad Prism software (Graphpad Prism).

3. Results

3.1. *IL-36α* is increased in the skin during IMQ-induced psoriatic dermatitis, but not hapten-induced CHS

The expression levels of IL-36α were increased in patients with psoriasis [17,22], and keratinocyte-derived IL-36α is known to be crucial for development of IMQ-induced psoriasis-like dermatitis in mice [23,24]. First, we used qPCR and immunohistochemical analysis to compare the expression levels of IL-36α in the inflamed skin of wild-type mice and *Il36a*^{−/−} mice (as negative controls) during IMQ-induced psoriasis-like dermatitis and FITC- and DNFB-induced CHS. In the wild-type mice, the qPCR analysis showed that the expression levels of *Il36a* mRNA were significantly increased in IMQ-treated ear skin compared with in vehicle-treated ear skin (Fig. 1A). On the other hand, the *Il36a* mRNA levels in FITC- and DNFB-treated ear skin of wild-type mice were comparable with those in vehicle-treated ear skin of wild-type mice and in the vehicle-, FITC- and DNFB-treated ear skin of *Il36a*^{−/−} mice (Fig. 1A). In the immunohistochemical analysis of wild-type mice, IL-36α was detected in keratinocytes of IMQ-treated ear skin (Fig. 1B), but hardly detectable in FITC- or DNFB-treated ear skin (Fig. 1B).

3.2. *IL-36α* is not essential for DC migration from the skin to draining LNs

IL-36α was shown to enhance mRNA expression for certain cytokines and chemokines in bone marrow cell-derived Langerhans cells (LCs) [25], suggesting that IL-36α acts on LCs. Therefore, we investigated for possible involvement of IL-36α in the ability of LCs to migrate from the skin to draining LNs. Twenty-four hours after epicutaneous treatment with FITC or vehicle alone, we determined the proportion of FITC-positive cells among MHC class II^{hi} CD11c⁺ cells in cervical LNs by flow cytometry. The proportion of FITC-positive cells in cervical LNs on the FITC-treated side was significantly increased compared with those on the vehicle-treated side in both wild-type and *Il36a*^{−/−} mice. However, the FITC-positive-cell proportion on the FITC-treated side was comparable between wild-type and *Il36a*^{−/−} mice (Fig. 2A and B). These observations indicate that IL-36α is not essential for DC migration from the skin to draining LNs.

3.3. *IL-36α* is involved in hapten-specific T-cell induction

IL-36α is expressed in activated DCs [25], and it can promote expression of co-stimulatory molecules such as CD80, CD86 and CD40 on DCs, and cytokine production by T cells [8]. These observations suggest that IL-36α may be involved in hapten-specific T-cell induction in the sensitization phase of CHS. Therefore, draining LN cells from wild-type and *Il36a*^{−/−} mice after sensitization with FITC or DNFB were cultured in the presence and absence of FITC or DNBS. We then determined the levels of IFN-γ, IL-4 and IL-17A in the culture supernatants and the proportions of IFN-γ⁺, IL-4⁺ and IL-17A⁺ cells in CD3⁺CD4⁺ cells and CD3⁺CD8⁺ cells in the cultures.

Concentrations of IFN-γ (A), IL-4 (B) and IL-17A (C) and the proportions of IFN-γ⁺ cells (A), IL-4⁺ cells (B) and IL-17A⁺ cells (C) in CD3⁺CD4⁺ cells and CD3⁺CD8⁺ cells in response to FITC. (D–F) Concentrations of IFN-γ (D), IL-4 (E) and IL-17A (F) and the proportions of IFN-γ⁺ cells (D), IL-4⁺ cells (E) and IL-17A⁺ cells (F) in CD3⁺CD4⁺ cells and CD3⁺CD8⁺ cells in response to DNBS. Data show the mean ± SEM of values for individual mice, and a representative result from two independent experiments. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001, medium alone vs. FITC or DNBS; and [†]*p* < 0.05 and ^{††}*p* < 0.001, LN cells from wild-type mice vs. LN cells from *Il36a*^{−/−} mice.

In FITC-specific LN-cell responses, the levels of IFN- γ and IL-4 and the proportions of IFN- γ^+ and IL-4 $^+$ cells were comparable between the wild-type and *Il36a* $^{-/-}$ mice (Fig. 3A and B). Although the levels of IL-17A in the culture supernatants were significantly decreased in the *Il36a* $^{-/-}$ mice compared with the wild-type mice (Fig. 3C), the proportion of IL-17A $^+$ cells in CD3 $^+$ CD4 $^+$ cells was significantly increased in the *Il36a* $^{-/-}$ mice compared with the wild-type mice, while their proportion in CD3 $^+$ CD8 $^+$ cells was comparable in both mice (Fig. 3C).

In DNP-specific LN-cell responses, the levels of IFN- γ and the proportions of IFN- γ^+ cells in CD3 $^+$ CD4 $^+$ cells and CD3 $^+$ CD8 $^+$ cells were significantly reduced in the *Il36a* $^{-/-}$ mice compared with the wild-type mice (Fig. 3D). The levels of IL-4 and the proportions of IL-4 $^+$ cells in CD3 $^+$ CD4 $^+$ cells and CD3 $^+$ CD8 $^+$ cells were also comparable in both mice, irrespective of the presence or absence of DNBS (Fig. 3E). Like the FITC-specific LN-cell responses, although

the levels of IL-17A in the culture supernatants were significantly decreased in the *Il36a* $^{-/-}$ mice compared with the wild-type mice (Fig. 3F), the proportion of IL-17A $^+$ cells in CD3 $^+$ CD4 $^+$ cells was significantly increased in the *Il36a* $^{-/-}$ mice. The proportion of IL-17A $^+$ cells in CD3 $^+$ CD8 $^+$ cells was also comparable in both mice, irrespective of the presence or absence of DNBS (Fig. 3F). These observations suggest that IL-36 α is involved in the induction and/or activation of hapten-specific T-cell subsets in the sensitization phase of FITC- and/or DNFB-induced CHS.

3.4. IL-36 α is not essential for induction of local inflammation during CHS

As shown above, IL-36 α is involved in induction of hapten-specific T-cell subsets in the sensitization phase. These observations suggest that IL-36 α may contribute to induction of local

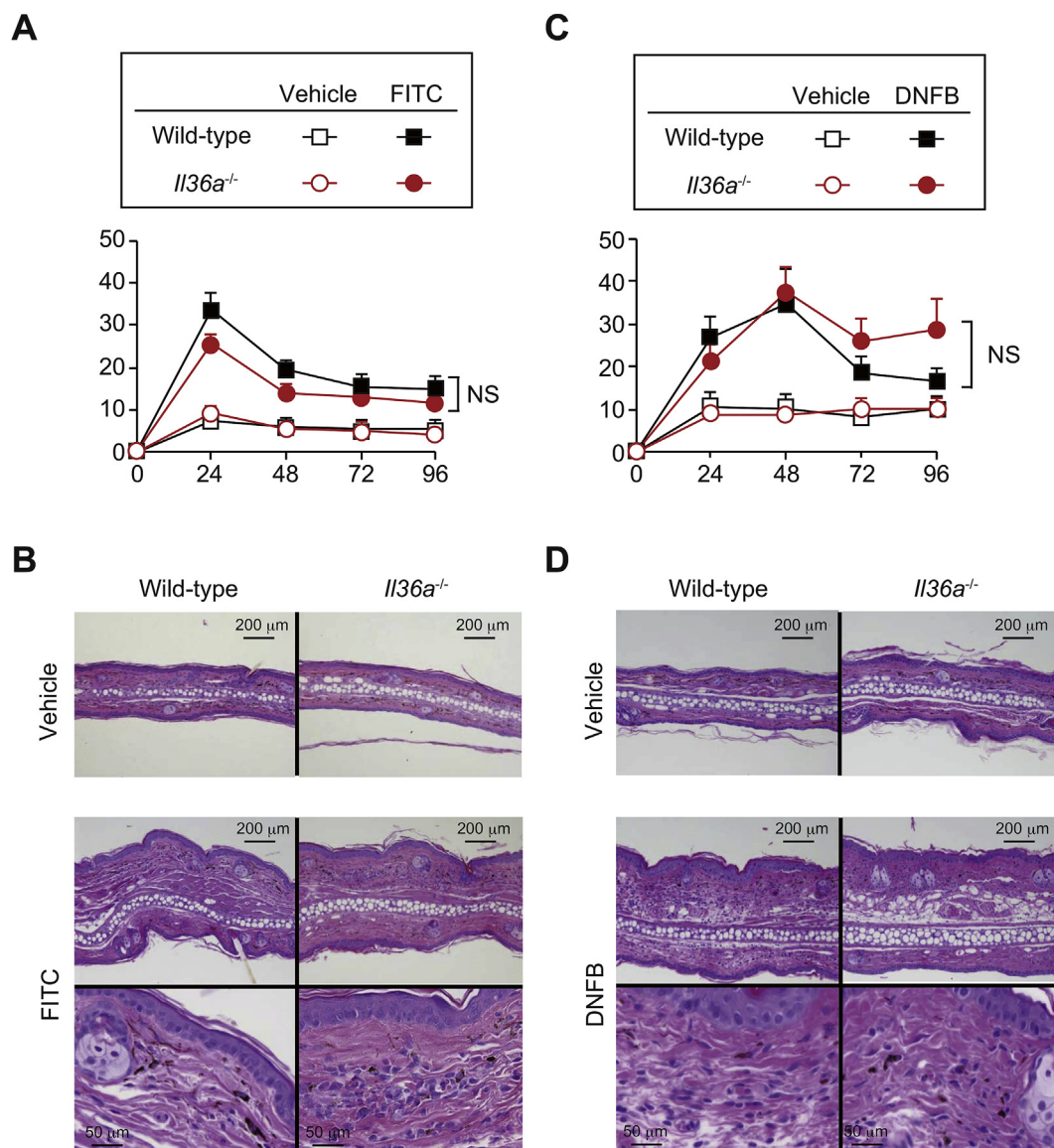


Fig. 4. IL-36 α is not essential for induction of local inflammation during FITC- or DNFB-induced CHS. Wild-type mice ($n = 10$) and *Il36a* $^{-/-}$ mice ($n = 10$) were epicutaneously sensitized and challenged with FITC or DNFB. The ear skin thickness of the mice was measured at the indicated time points after challenge with FITC or vehicle alone. (A) Sections of ear skin from the mice 24 h after challenge with FITC or vehicle alone were stained with hematoxylin and eosin. (B) Wild-type mice ($n = 10$) and *Il36a* $^{-/-}$ mice ($n = 10$) were epicutaneously sensitized and challenged with DNFB. The ear skin thickness of the mice was measured at the indicated time points after challenge with DNFB or vehicle alone. (C) Sections of ear skin from the mice 24 h after challenge with DNFB or vehicle alone were stained with hematoxylin and eosin. Data show the mean $\pm$ SEM, and a representative result from two independent experiments (A, C).

inflammation in the elicitation phase of CHS via activation of hapten-specific T-cell subsets. However, the local inflammation—assessed by measurement of ear skin thickness—was comparable between wild-type and *Il36a*^{−/−} mice during FITC-induced CHS (Fig. 4A). Consistent with this, at 24 h after challenge with FITC, the degree of skin inflammation—assessed by histological analysis—was also identical in both mice (Fig. 4B). The development of DNFB-induced CHS was also similar in both mice (Fig. 4C and D). These observations indicate that IL-36 α is not essential for induction of local inflammation during either FITC- or DNFB-induced CHS.

4. Discussion

Several lines of evidence demonstrated increased expression of IL-36 in specimens from patients with psoriasis [20,21]. In addition, IL-36 α , but not IL-36 β or IL-36 γ , is crucial for development of IMQ-induced murine psoriatic dermatitis [23–25]. Compared with the role of IL-36 in psoriasis, the contribution of this cytokine to development of ACD/CHS is poorly understood. In the present study, using *Il36a*^{−/−} mice, we clearly demonstrated that IL-36 α is somewhat involved in induction and/or activation of hapten-specific T-cell subsets in the sensitization phase, but is not involved in induction of local inflammation in the elicitation phase, of FITC- and DNFB-induced CHS.

Expression of IL-36 α , IL-36 β and IL-36 γ was increased in skin biopsies of patients with nickel-mediated ACD [18]. In contrast, expression of IL-36 α was not increased in the inflamed skin of mice during FITC- or DNFB-induced CHS (Fig. 1). This apparent discrepancy may be due to the difference in haptens. It is known that the phenotypes of CHS differ with use of different haptens and vehicles, even in the same strain of mice [28].

IL-36 α is expressed in mouse bone-marrow-cell-derived DCs in response to certain stimuli [24,25]. Moreover, IL-36 α can activate such DCs to express co-stimulatory molecules, cytokines and chemokines [8,25]. In association with this, DC-derived IL-36 α and IL-36 α -mediated DC activation are suggested to contribute to development of IMQ-induced psoriatic dermatitis in mice [25]. In addition, IL-36 is known to enhance proliferation, as well as secretion of cytokines such as IFN- γ , IL-4 and IL-17, by CD4⁺ and/or CD8⁺ T cells [8,11–13]. These observations suggest that IL-36 α , which is derived from DCs, may enhance activation of T cells directly and indirectly via activation of DCs during antigen presentation by DCs in LNs. Although we found that IL-36 α was not essential for skin DC function, i.e., migration of DCs from the skin to draining LNs, this cytokine is involved in induction and/or activation of hapten-specific T-cell subsets in the sensitization phase of CHS. In the sensitization phase of DNFB-induced CHS, but not FITC-induced CHS, IFN- γ production by LN cells in response to DNBS and the proportions of IFN- γ ⁺ CD4⁺ T cells and IFN- γ ⁺ CD8⁺ T cells in LN cells were significantly increased in *Il36a*^{−/−} mice compared with wild-type mice (Fig. 3D). Thus, consistent with a previous report [11], IL-36 α is also important for induction and activation of Th1 cells and Tc1 cells in the sensitization phase of DNFB-induced CHS, but not FITC-induced CHS. In the sensitization phase of FITC- and DNFB-induced CHS, IL-17A production by LN cells in response to FITC and DNBS was significantly reduced in *Il36a*^{−/−} mice compared with wild-type mice (Fig. 3C and F). Nevertheless, the proportion of IL-17A⁺ CD4⁺ T cells in LN cells was significantly increased in *Il36a*^{−/−} mice compared with wild-type mice (Fig. 3C and F). In the setting, IL-36 α has an inhibitory role in differentiation of Th17 cells, but a facilitative role in activation of Th17 cells. Although we demonstrated that IL-36 α was involved in induction and/or activation of hapten-specific T-cell subsets in the sensitization of FITC- and DNFB-induced CHS, it was not essential for induction of local

inflammation in the skin in the elicitation phase. In association with this, IL-36 α expression was hardly detectable in the inflamed skin during either FITC- or DNFB-induced CHS (Fig. 1). These observations suggest that IL-36 β and/or IL-36 γ may play compensatory roles for IL-36 α in induction of local inflammation in the elicitation phase of CHS. The roles of IL-36 β and IL-36 γ in development of CHS should be elucidated in future studies.

In summary, IL-36 α is involved in induction and/or activation of hapten-specific T-cell subsets in the sensitization phase of CHS, but it is not essential for induction of local inflammation in the elicitation phase of CHS.

Conflicts of interest

The authors have no conflicts of interest to declare.

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