

Thymic stromal lymphopoietin-activated invariant natural killer T cells trigger an innate allergic immune response in atopic dermatitis

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Background: Although invariant natural killer T (iNKT) cells have been shown to play a critical role in the pathogenesis of asthma, the role of iNKT cells in atopic dermatitis (AD) has not been well evaluated.

Objective: We investigated whether iNKT cells in patients with AD increased and whether iNKT cells were activated by thymic stromal lymphopoietin (TSLP), which is highly expressed in keratinocytes of AD.

Methods: We assessed the population of iNKT cells in PBMCs of patients with AD and healthy controls (HCs) using flow cytometry. Immunohistochemistry was used to evaluate iNKT cells and TSLP expression in AD and HC skin. We also evaluated whether iNKT cells expressed the TSLP receptor, the effects of TSLP on iNKT cells, and iNKT cell-dendritic cell interactions in a TSLP-rich environment.

Results: There were more iNKT cells among PBMCs of patients with moderate to severe AD than mild AD ($P < .05$) and HC ($P < .001$). The number of iNKT cells was significantly larger in severe AD skin lesions than in mild ($P < .001$) or moderate AD skin lesions ($P < .05$). TSLP expression increased in lesional skin ($P < .001$) but not in the sera of patients with AD ($P = .729$) compared with HC. iNKT cells expressed TSLP receptor protein and mRNA. TSLP directly activated iNKT cells to secrete IL-4 and IL-13, and the concurrent addition of dendritic cells further activated IFN- γ expression.

Conclusion: Increased iNKT cells activated by TSLP, especially in patients with severe AD, might play an essential role in the innate allergic immune response in AD. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: Atopic dermatitis, invariant natural killer T cells, NKT cells, thymic stromal lymphopoietin, dendritic cells

Abbreviations used

AD:	Atopic dermatitis
α -GalCer:	α -Galactosylceramide
DC:	Dendritic cell
EASI:	Eczema Area and Severity Index
FITC:	Fluorescein isothiocyanate
HC:	Healthy control
HPF:	High-power field
IDEC:	Inflammatory dendritic epidermal cell
IL-7R α :	IL-7 receptor α -chain
iNKT:	Invariant natural killer T
NKT:	Natural killer T
PE:	Phycoerythrin
TCR:	T-cell receptor
TSLP:	Thymic stromal lymphopoietin
TSLPR:	Thymic stromal lymphopoietin receptor chain

Atopic dermatitis (AD) is a highly pruritic, chronic relapsing inflammatory skin disease, characterized by skin barrier dysfunction resulting in dry skin and allergic sensitization to food and environmental antigens.^{1,2} The most common form of AD, accounting for 70% to 80% of cases, is allergic AD with elevated concentrations of total and allergen-specific IgE in serum and skin.³ The remaining 20% to 30% patients have nonallergic AD, with normal total IgE levels and negative serum allergen-specific IgE, reflecting multiple different pathogenic mechanisms and indicating that AD represents a collection of heterogeneous groups.⁴

Natural killer T (NKT) cells represent a subpopulation of thymus-derived T cells that coexpress a T-cell receptor (TCR) along with typical surface receptors for natural killer cells.⁵ More than 80% of NKT cells express invariant TCR- α chain V α 24-J α 18 paired with V β 11 in human beings (or V α 14-J α 18 paired with V β 8, V β 7, or V β 2 in mice) and are referred to as invariant NKT (iNKT) cells.⁶ iNKT cells uniquely recognize glycolipid antigens presented by the nonpolymorphic MHC class I-like molecule CD1d. A glycolipid isolated from a marine sponge, α -galactosylceramide (α -GalCer), binds CD1d and activates mouse and human iNKT cells,⁷ but the natural ligands presented *in vivo* by CD1d to iNKT cells remain unclear except for a lysosomal glycosphingolipid, isoglobotrihexosylceramide.⁸ iNKT cells rapidly produce large quantities of cytokines, including IL-4 and IFN- γ , as a manifestation of innatelike immunity. This rapid production of cytokines regulates adaptive immune responses by activation of dendritic cells (DCs), natural killer cells, B cells, and conventional T cells, thus linking innate and adaptive immunity.⁹ iNKT cells play a critical role in mouse asthma models and

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Supported by a grant of the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (A080892).

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication September 24, 2009; revised May 8, 2010; accepted for publication May 21, 2010.

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doi:10.1016/j.jaci.2010.05.024

patients with asthma.¹⁰ The role of iNKT cells in AD, however, has not been well investigated. Furthermore, contradictory results in peripheral blood of patients with AD have been reported.¹¹

Thymic stromal lymphopoietin (TSLP) is an epithelial cell-derived cytokine produced by human keratinocytes in AD.¹² TSLP exerts its biological activities by binding to a heterodimeric receptor consisting of the IL-7 receptor α -chain (IL-7R α) and the TSLP receptor chain (TSLPR).¹³ TSLP not only triggers adaptive allergic immune responses by stimulating myeloid DCs to induce T_H2-cell responses but also rapidly induces an innate type of allergic immune response by activating mast cells and NKT cells.¹⁴ The finding that TSLP may potentially activate NKT cells to produce IL-13 in a mouse asthma model¹⁵ suggests that TSLP may activate iNKT cells to play distinct roles in the pathogenesis of AD in human beings. Here we investigated whether iNKT cell number and TSLP expression were greater in patients with AD, whether iNKT cells expressed TSLP receptor, and whether these cells were activated by TSLP.

METHODS

Subjects

Blood samples were obtained from 10 nonatopic healthy controls (HCs; 6 men, 4 women; age 21–23 years) and 25 patients with AD (16 males, 9 females; age 15–37 years) as defined by the criteria of Hanifin and Rajka¹⁶ (see this article's Table E1 in the Online Repository at www.jacionline.org). Skin biopsy specimens were taken from the patients with AD ($n = 25$) and some of the HCs ($n = 5$). AD clinical severity was documented independently and blindly by the clinicians using the Eczema Area and Severity Index (EASI).¹⁷ We divided the patients with AD into 3 groups according to their EASI score (mild, <10; moderate, 10–20; severe, >20).¹⁸ The patients did not receive any systemic or topical treatment with immunosuppressive drugs for at least 4 weeks before collection of blood samples and skin biopsies. For functional studies using sorting of iNKT cells and coculture of iNKT cells–DCs, PBMCs were obtained by Ficoll fractionation of normal blood donor buffy coat preparations. All subjects provided written informed consent to participate in the study, and the institutional review board approved this study.

Flow cytometry and sorting

For surface staining, cells were stained with fluorescein isothiocyanate (FITC)–antihuman V α 24 mAb (Beckman Coulter, Fullerton, Calif), FITC–antihuman 6B11 mAb directed against the complementarity-determining-region 3 of the V α 24-J α 18 TCR of iNKT cells (BD Biosciences, San Jose, Calif), phycoerythrin (PE)–antihuman V β 11 mAb (Beckman Coulter), PE–antihuman CD3 mAb (BD Biosciences), PE–antihuman TSLPR mAb, and allophycocyanin–antihuman IL-7R α mAb (eBioscience, San Diego, Calif) per the manufacturers' standard protocols. For intracellular staining, cells were fixed and permeabilized with Cytofix/Cytoperm solution (eBioscience), then stained with PE–antihuman IL-4, IL-13, and IFN- γ mAbs (BD Biosciences). Cells were treated with 1, 10, 50, or 100 ng/mL TSLP and 10 μ g/mL antihuman TSLPR-neutralizing antibody (R&D Systems, Minneapolis, Minn) for 24 hours. Flow cytometry was performed by using a FACSCalibur (Becton Dickinson, Mountain View, Calif), then compensated and analyzed with WINMDI software (Scripps Research Institute, San Diego, Calif).

For sorting of iNKT cells, isolated PBMCs from normal blood donor buffy coat preparations were washed and incubated with FITC–antihuman 6B11 mAb. Cells were sorted with an EPIC Altra cell sorter (Beckman Coulter). The purity of selected iNKT cells was >98% in all cases. Sorted cells were incubated with 1 or 50 ng/mL TSLP and 100 ng/mL α -GalCer (Biomol International, Plymouth Meeting, Pa) for 24 hours.

Immunohistochemical staining for TSLP and V α 24

Acetone-fixed 6- μ m cryostat sections were stained with mouse anti-TSLP (R&D Systems), anti-V α 24 (Beckman Coulter), anti-CD3 mAb, or isotype

control antibody (BD Pharmingen, San Diego, Calif). Double staining was performed by using anti-V α 24 and anti-CD3 mAbs. Development of the staining was performed with a Histostatin-DS kit (ZYMED, San Francisco, Calif) according to the manufacturer's recommendations. The intensity of TSLP staining was graded under microscopy on a scale from 0 (no staining) to 6 (the most intense staining). iNKT cell numbers were determined by calculating the average number of V α 24-positive cells per high-power field (HPF; $\times 400$) and summed for the selected 5 different areas containing the largest number of iNKT cells. Two independent investigators evaluated staining intensity in a blind manner.

ELISA

Secreted cytokine levels for IL-4, IL-13, and IFN- γ in the supernatants from sorted iNKT cells were quantitated by using commercially available ELISA kits (eBioscience) according to the manufacturer's instructions.

RT-PCR and Western blot analysis for TSLP receptor

Total RNA was extracted from sorted 6B11⁺ iNKT cells and remnant 6B11[−] cells by using an RNeasy mini kit (Qiagen, Valencia, Calif), and cDNA was synthesized with an oligo dT primer after deoxyribonuclease treatment. The following primers were used: TSLPR, 5'-CTG ACC TGT CCT ACG GGG AT-3' (sense) and 5'-CCT GGA AGT TCC CTT GGT GT-3' (antisense); IL-7R α , 5'-ACT ACA AAC AGC ACG CTG CC-3' (sense) and 5'-TTC CTG CCT TCC TCA CTC CT-3' (antisense); and β -actin, 5'-GGC GGA CTA TGA CTT AGT TG-3' (sense) and 5'-AAA CAA CAA TGT GCA ATC AA-3' (antisense). The number of PCR cycles was 38.

Western blotting was carried out by using 30 μ g protein from whole-cell lysates of 6B11⁺ iNKT cells and 6B11[−] cells. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, Calif) and detected by using ECL (Amersham Bioscience, Arlington Heights, Ill). Goat antihuman TSLPR (R&D Systems) and mouse antihuman IL-7R α (Dendritics, Lyon, France) antibodies were used at a dilution of 1:500. Mouse anti- β -actin antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) was used as a loading control at 1:5000.

Culture of DCs and coculture of iNKT cells and DCs

The culture media consisted of RPMI 1640 supplemented with 100 IU/mL penicillin, 100 μ g/mL streptomycin (Gibco Laboratories, Grand Island, NY), and 10% FBS (Hyclone, Logan, Utah). Monocytes were isolated from PBMCs from normal blood donor buffy coat preparations via the cell attachment method.¹⁹ PBMCs were attached to 6-well plates for 1 hour in RPMI 1640 with 3% inactivated human plasma. The supernatant and any floating cells were discarded, and the attached cells were used for culture. Monocytes were plated at a final concentration of 3×10^6 cells in 3 mL culture medium. DCs were generated by culturing monocytes for 6 days in medium supplemented with 500 U/mL GM-CSF and 1000 U/mL IL-4 (R&D Systems) at days 0, 2, 4, and 6. At days 2, 4, and 6, one third of the medium was removed, and an equivalent volume of fresh cytokine-enriched medium was added. The purity of the DCs was >98% CD1a⁺. DCs were stained with FITC–antihuman CD1d mAb (BD Pharmingen). DCs were cocultured with iNKT cells at 1×10^6 and 1×10^5 cells per well, respectively, for 24 hours at 37°C in 10% RPMI 1640 with 1 or 50 ng/mL TSLP and 100 ng/mL α -GalCer.

Statistical analysis

All statistical analysis was conducted by using SPSS version 12.0 software (SPSS, Inc, Chicago, Ill). Data were analyzed with the Mann-Whitney *U* test. Correlations were determined by calculating the Pearson correlation coefficient. *P* < .05 was considered statistically significant.

RESULTS

iNKT cells increase in PBMCs of moderate to patients with severe AD

Invariant natural killer T cells were identified as V α 24⁺/CD3⁺, V α 24⁺/V β 11⁺, or 6B11⁺/V β 11⁺ cells (Fig 1, A). In PBMCs of

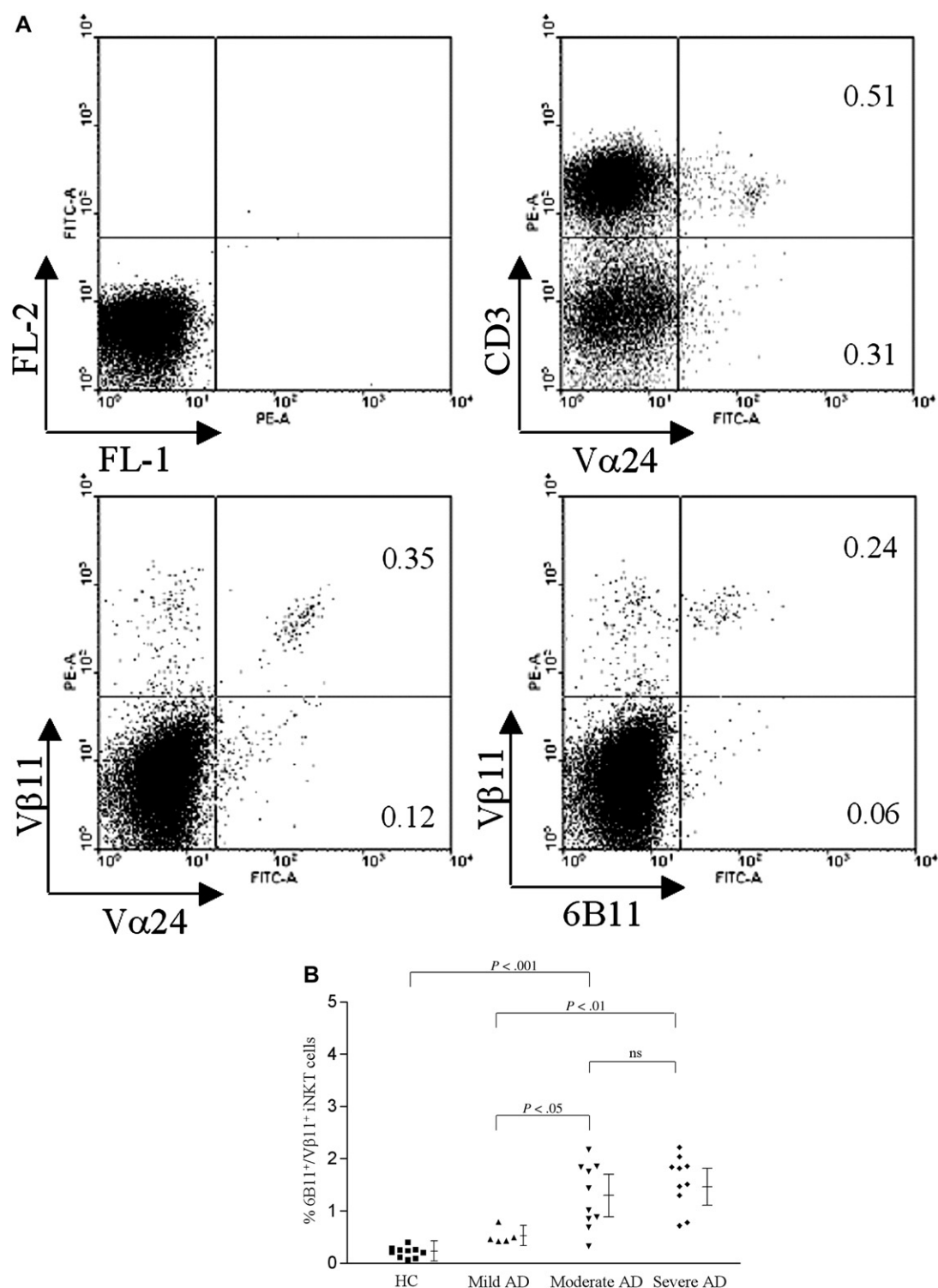


FIG 1. iNKT cells in PBMCs of HCs and patients with AD. **A**, iNKT cells were identified as Vα24⁺/CD3⁺, Vα24⁺/Vβ11⁺, or 6B11⁺/Vβ11⁺ cells. **B**, The percentage of 6B11⁺/Vβ11⁺ iNKT cells in PBMCs of HCs (n = 10) and patients with mild (n = 5), moderate (n = 10), and severe AD (n = 10) was evaluated by using flow cytometry. FL, Fluorescence; ns, not significant.

HCs and patients with AD, iNKT cells were defined as Vα24⁺/Vβ11⁺ or 6B11⁺/Vβ11⁺ cells because these populations were more specific than Vα24⁺/CD3⁺ cells. Results were similar using either set of cell markers, but there were slightly more Vα24⁺/

Vβ11⁺ cells (data not shown) than 6B11⁺/Vβ11⁺ cells (Fig 1, B). PBMCs from patients with AD contained a greater percentage of iNKT cells than HCs (mean ± SD, 1.13 ± 0.62 vs 0.21 ± 0.10; P < .001). Although the number of iNKT cells in PBMCs of

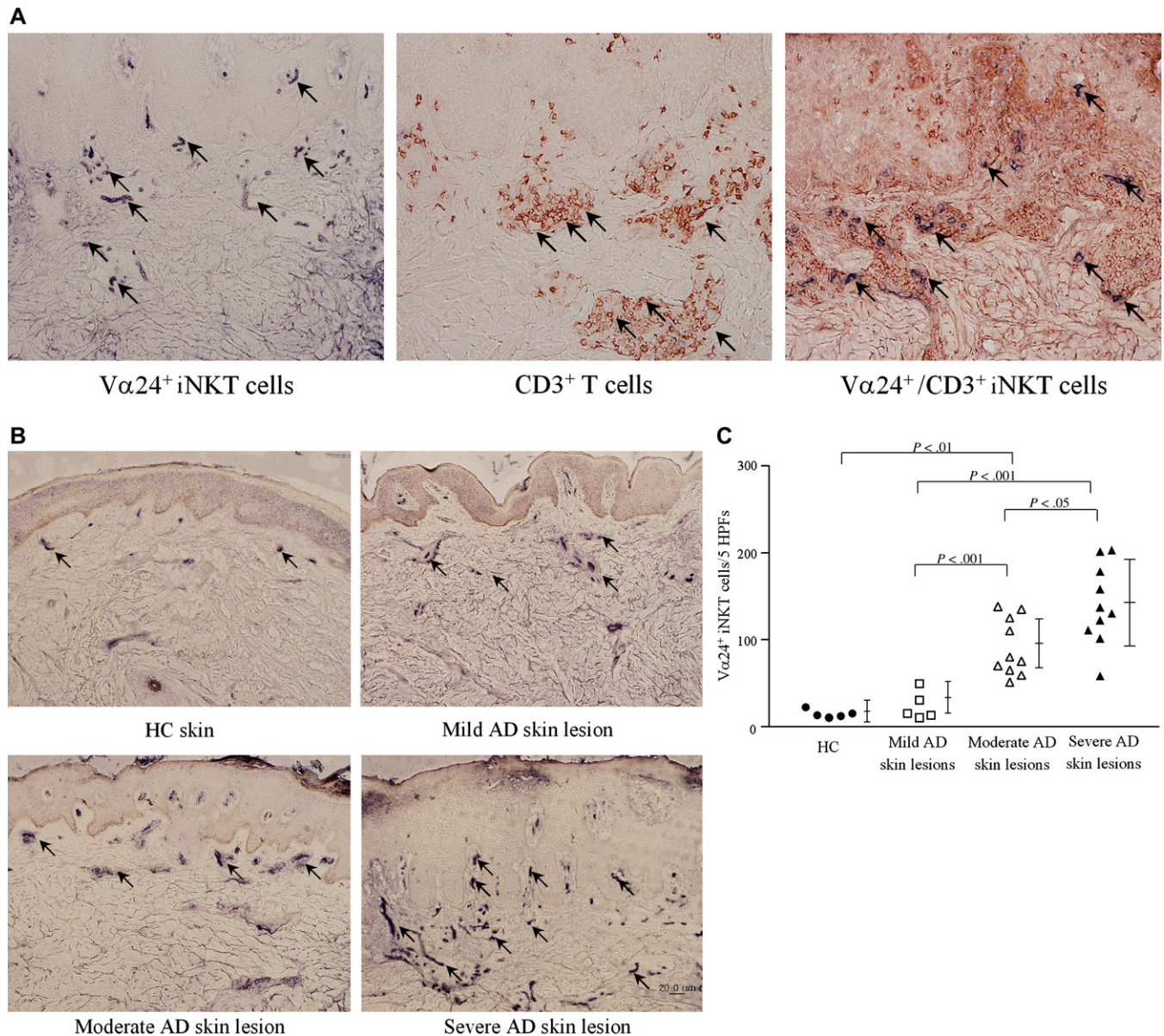


FIG 2. iNKT cell numbers increase in the lesional skin of patients with AD. **A**, Vα24⁺ iNKT cells were present among CD3⁺ T-cell infiltration in AD skin. Original magnification $\times 200$. **B**, Representative immune-stained skin sections from HCs and patients with mild, moderate, and severe AD are shown. Original magnification $\times 100$. Arrows indicate Vα24⁺ iNKT cells. **C**, The number of Vα24⁺ iNKT cells (cells/5 HPFs) in skin sections from HCs ($n = 5$) and mild ($n = 5$), moderate ($n = 10$), and severe AD skin lesions ($n = 10$).

patients with moderate (1.28 ± 0.61) and severe AD (1.55 ± 0.50) were not significantly different from each other, the percentage of iNKT cells in patients with moderate to severe AD was significantly larger than that of patients with mild AD (0.51 ± 0.15 ; $P < .05$, $P < .01$, respectively). We did not find any correlation between iNKT cell numbers and serum IgE levels (see this article's Fig E1, A, in the Online Repository at www.jacionline.org).

iNKT cells increase in lesional skin of patients with severe AD

A large number of CD3⁺ T cells infiltrated the dermis of AD skin, along with Vα24⁺ iNKT cells among CD3⁺ T cells (Fig 2, A). iNKT cells were present in HC skin (14.4 ± 4.6 cells/5

HPFs), but significantly more iNKT cells infiltrated the dermis of AD skin lesions (97.0 ± 56.2), correlating with the degree of AD severity (Fig 2, B and C). iNKT cell numbers were larger in severe AD skin lesions (139.9 ± 45.9) than in mild (23.4 ± 16.3 ; $P < .001$) or moderate AD skin lesions (90.8 ± 33.0 ; $P < .05$). There was no correlation between skin iNKT cell infiltration and serum IgE levels in patients with AD (Fig E1, B).

TSLP expression increases in lesional skin but not in sera of patients with AD

Nonatopic HC skin had minimal TSLP in the epidermis, whereas the epidermis of AD skin lesions contained high levels of TSLP (see this article's Fig E2, A, in the Online Repository at

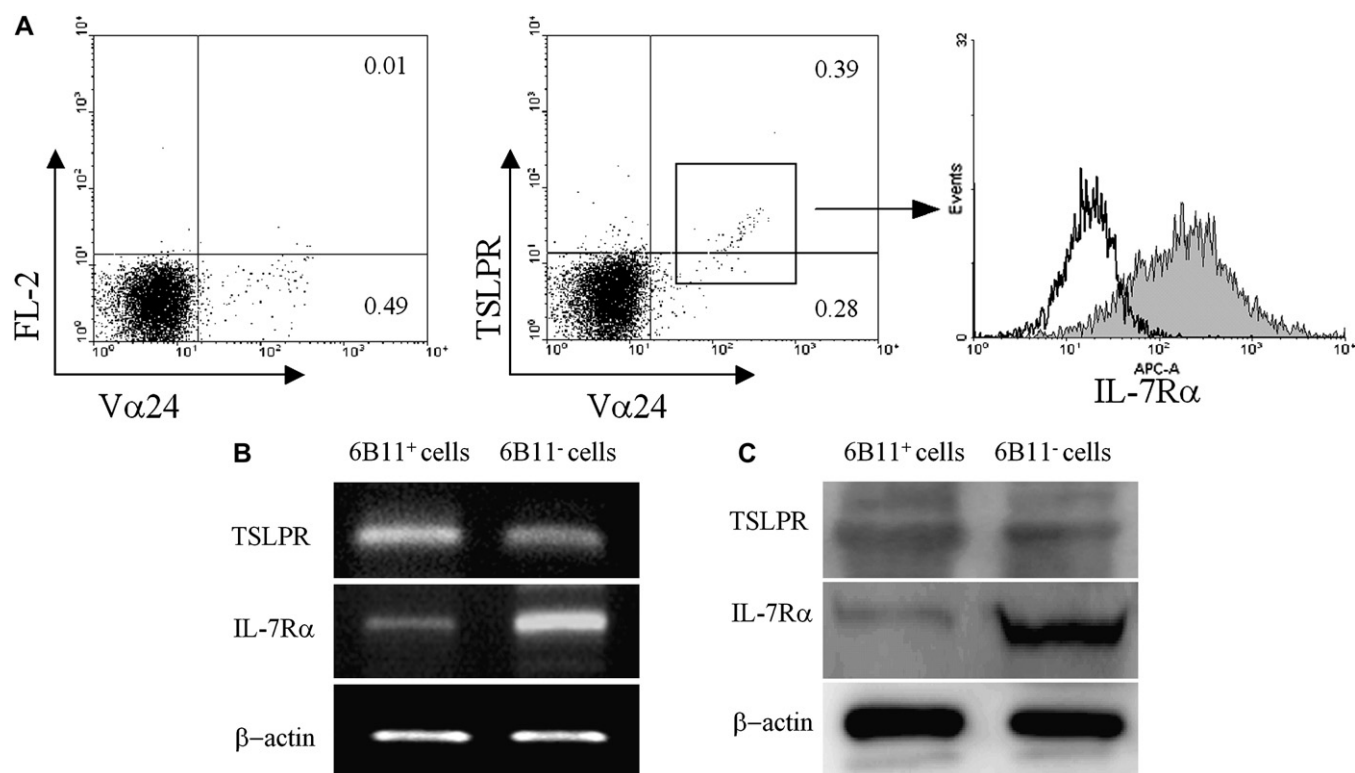


FIG 3. TSLP receptor expression in iNKT cells. **A**, TSLPR and IL-7Rα protein expression in Vα24⁺ iNKT cells in normal PBMCs. Sorted 6B11⁺ iNKT cells from a normal donor expressed both TSLPR and IL-7Rα mRNA (**B**) and protein (**C**). FL, Fluorescence.

www.jacionline.org). There was, however, no significant difference in TSLP expression in skin lesions of patients with AD according to the degree of AD severity (Fig E2, B). In contrast with the high TSLP expression in the epidermis of AD, we could scarcely detect TSLP protein in the sera of patients with AD or HCs (9.53 ± 3.54 vs 9.83 ± 3.40 pg/mL; Fig E2, C). There was no correlation between TSLP expression and serum IgE levels in patients with AD (Fig E1, C). There was no statistically significant correlation between serum IgE levels and the EASI scores of the patients, although patients with severe AD had non-significantly higher levels of serum IgE than patients with mild or moderate AD (Fig E1, D).

TSLP directly activates iNKT cells to secrete IL-4 and IL-13 but also produce IFN-γ in concurrent coculture with DCs

To examine whether TSLP has a direct effect on iNKT cells, we first demonstrated that Vα24⁺ iNKT cells expressed the TSLP receptor consisting of TSLPR and IL-7Rα in PBMCs from a normal donor (Fig 3, A). We sorted iNKT cells by using the 6B11 mAb because this cell marker is more specific than Vα24 mAb for identification of iNKT cells on the basis of our results and a previous report.²⁰ The sorted 6B11⁺ iNKT cells expressed both TSLPR and IL-7Rα mRNA and protein (Fig 3, B and C). 6B11⁻ cells expressed higher levels of IL-7Rα mRNA and protein than the 6B11⁺ iNKT cells, implying that other cells, such as conventional T cells, B cells, and monocytes, express IL-7 receptor at high levels.

Normal PBMCs were incubated with 1, 10, 50, or 100 ng/mL TSLP and 10 μg/mL antihuman TSLPR neutralizing antibody to evaluate dose-dependent responses to TSLP and the blocking effect of TSLPR on iNKT cells. Vα24⁺ iNKT cells treated with 50 ng/mL TSLP expressed the peak level of IL-4. However, IL-13 expression was enhanced in the Vα24⁺ iNKT cells treated with only 1 ng/mL TSLP. TSLP-treated Vα24⁺ iNKT cells produced a higher level of IL-4 than IL-13. Neutralization of TSLPR reduced IL-4 expression by 55% and IL-13 expression by 58% compared with 50 or 1 ng/mL TSLP treatment, respectively. IFN-γ expression was not affected by TSLP treatment (Fig 4, A and B). These data imply that TSLPR is required for optimal IL-4 and IL-13 expression in TSLP-treated iNKT cells. We also confirmed that sorted iNKT cells from normal PBMCs treated with TSLP and iNKT cells in lesional skin from patients with AD expressed both IL-4 and IL-13 using confocal laser microscopy (Fig 4, C).

For functional studies, sorted iNKT cells were treated with 1 or 50 ng/mL TSLP or 100 ng/mL α-GalCer for 24 hours. IL-4 and IL-13, but not IFN-γ, expression increased in the TSLP-treated iNKT cells, although α-GalCer-treated iNKT cells expressed high levels of IL-4, IL-13, and IFN-γ (Fig 5, A). Cytokine levels in the supernatants from iNKT cells showed a similar pattern, but α-GalCer-treated iNKT cells secreted lower levels of these cytokines (Fig 5, B). These results suggest that TSLP might directly activate iNKT cells into a T_H2 pattern in allergic inflammation.

Next, we examined the interaction between DCs and iNKT cells in a TSLP-rich environment by using a coculture method. IL-4 and IL-13 expression in iNKT cells cocultured with DCs in

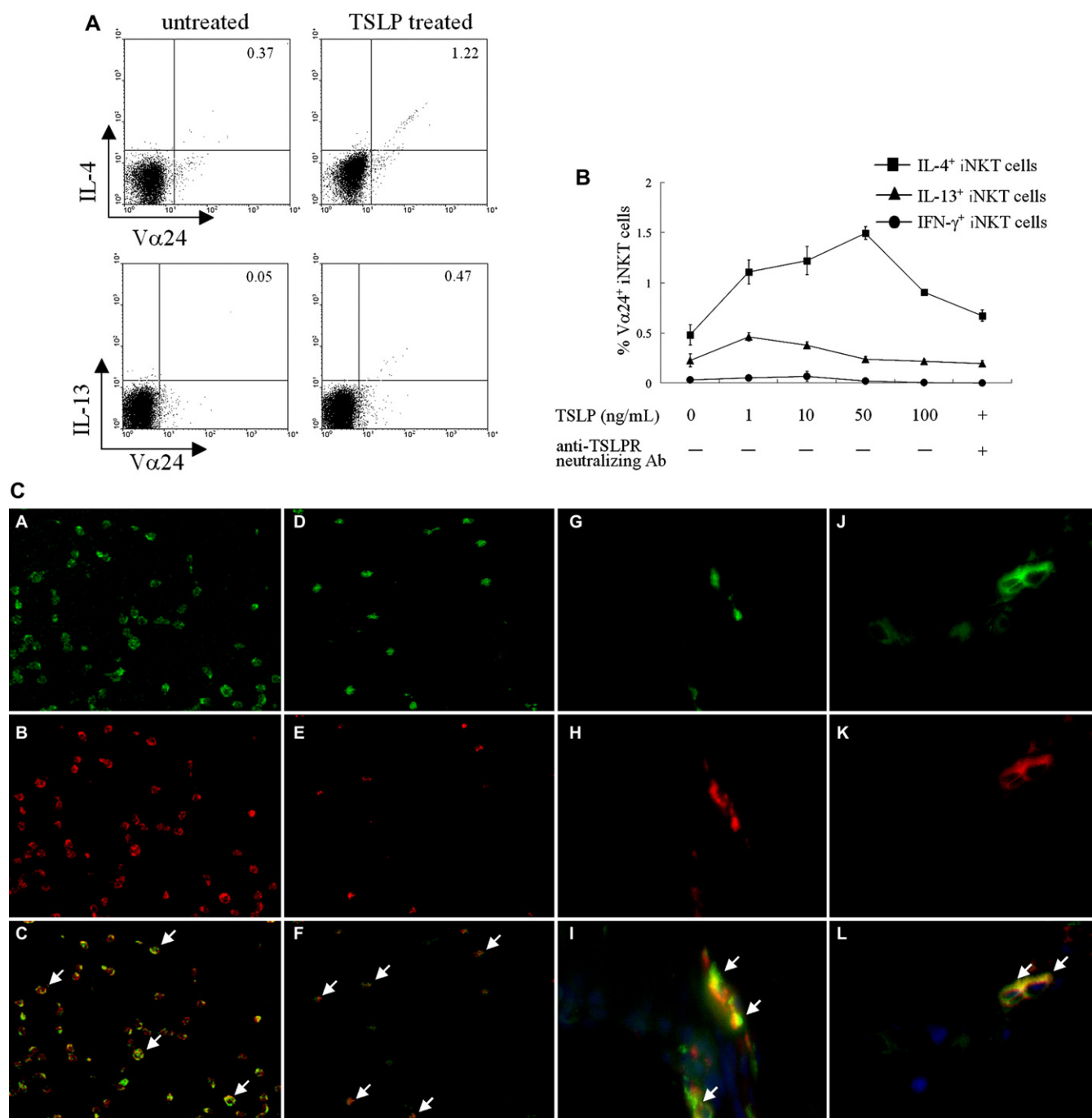


FIG 4. A, Flow-cytometric analysis of IL-4 and IL-13 in $V\alpha 24^+$ iNKT cells in normal PBMCs treated with TSLP. B, Dose-dependent response to TSLP and neutralization effect of the anti-TSLPR Ab on $V\alpha 24^+$ iNKT cells. C, Confocal laser microscopy of purified iNKT cells from PBMCs treated with TSLP (A and D) and iNKT cells in lesional skin of patients with AD (G and J) expressing IL-4 (B and H) or IL-13 (E and K). Arrows indicate colocalization of iNKT cells with IL-4 (C and I) or IL-13 (F and L). Skin sections were counterstained with 4',6-diamidino-2-phenylindole. Data shown are representative of 3 independent experiments.

TSLP-rich media was higher than in those cocultured with DCs without TSLP-rich media. This expression pattern was the same as in TSLP-treated iNKT cells alone. Cocultured iNKT cells treated with TSLP expressed significantly more IFN- γ than did iNKT cells treated with only TSLP or DCs. α -GalCer-treated iNKT cells cocultured with DCs expressed high levels of all of

the cytokines (Fig 5, C). To confirm the interaction between DCs and iNKT cells, we investigated whether DCs express the CD1d molecule to interact with the invariant TCR of iNKT cells. CD1d was expressed on the surface of DCs (Fig 5, D), and TSLP or α -GalCer did not affect its expression (data not shown).

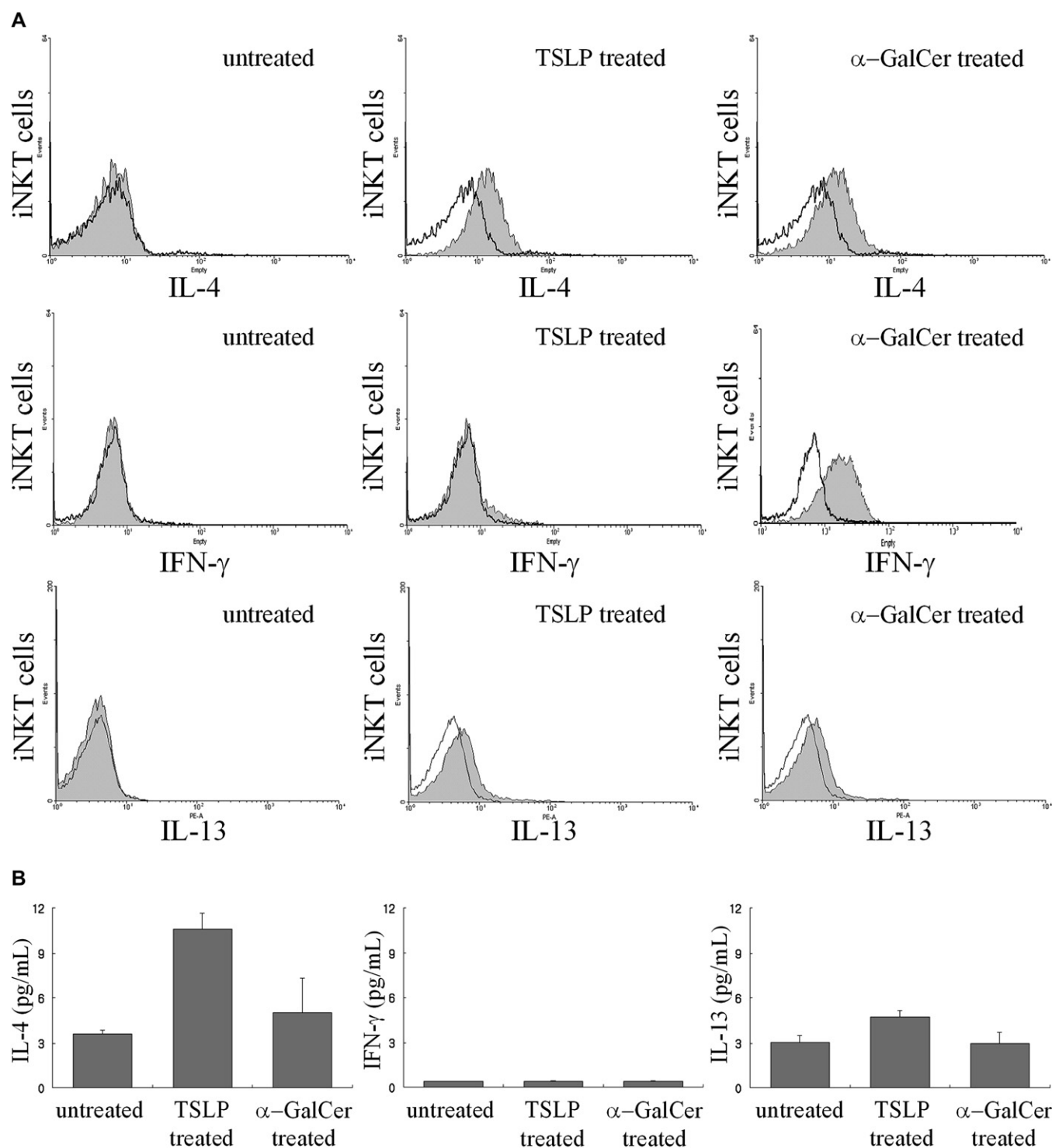


FIG 5. Cytokine expression in the iNKT cells (**A**), secreted cytokine levels in the supernatants from the iNKT cells (**B**), and cytokine expression in the iNKT cells cocultured with DCs (**C**) treated with TSLP or α -GalCer. Differing concentrations of TSLP were used (50 ng/mL for IL-4 or IFN- γ expression and 1 ng/mL for IL-13 expression). **D**, DCs expressed CD1d. Data shown are representative of 3 independent experiments.

DISCUSSION

Recent progress in allergic immunology has begun to discriminate between innate allergic inflammation versus innate defense mechanisms in the innate immunity of allergic diseases. Innate

defense immune responses, including pathogen-associated molecular pattern molecules and antimicrobial peptides expressed by keratinocytes, play important roles in protecting the skin against infection. Some of them are altered in patients with AD, who are

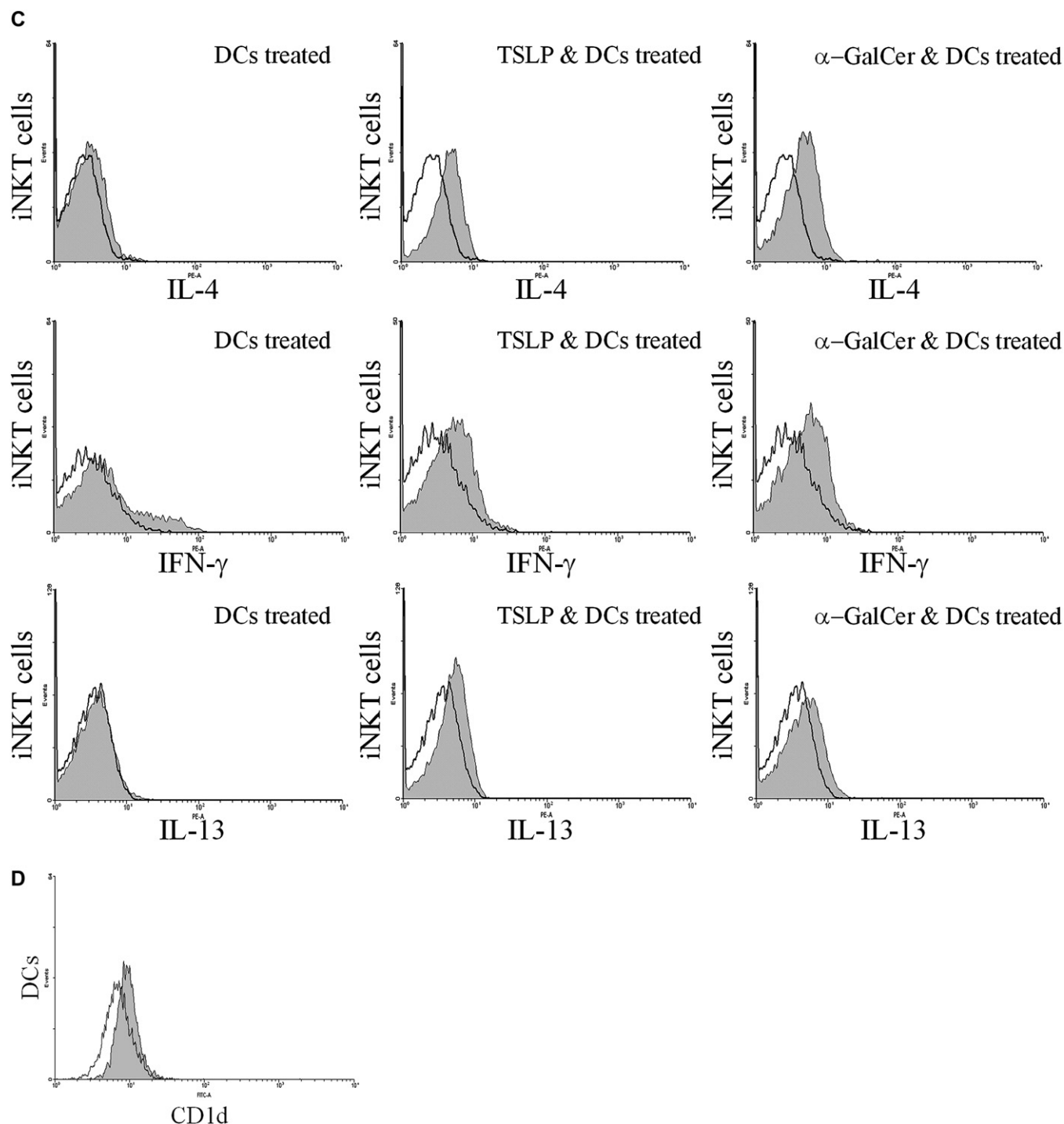


FIG 5. (Continued)

susceptible to bacterial and viral infections, particularly with *Staphylococcus aureus*, herpes simplex virus, and vaccinia virus.²¹ On the other hand, the findings that TSLP can initiate and aggravate allergic inflammation without the adaptive immune response, such as T cells and IgE, using TSLP-transgenic mice,^{22,23} and that TSLP also potently activates innatelike mast cells to produce high levels of T_H2 cytokines²⁴ imply a critical role for the innate allergic immune response in the pathogenesis of allergic diseases.

In addition to their rapid effector response and lack of immune memory, a conserved role for iNKT cells as innate T cells in innate immunity is suggested by the findings that the TCR of iNKT cells, which is conserved across phylogeny, may have specifically evolved as a pattern-recognition receptor, and that recognition of CD1d-associated glycolipids by iNKT cells is highly conserved.²⁵ Recent reports indicate that iNKT cells contribute to innate allergic immune responses by producing IL-4 and

IL-13 during the development of asthma in mice and human beings.²⁶⁻²⁸ Chronic lung disease, including asthma, arises independent of an adaptive immune response and is driven instead by an innate immune axis that relies on iNKT cells programmed to activate macrophages to produce IL-13.²⁹

We found that there were more $V\alpha 24^+/V\beta 11^+$ or $6B11^+/V\beta 11^+$ iNKT cells in PBMCs of patients with AD compared with HCs, in agreement with 1 previous study.³⁰ Others have reported, however, that iNKT cell levels in the peripheral blood of patients with AD are lower or not significantly different than HCs.³¹⁻³⁵ Elevated iNKT cells in PBMCs of patients with AD may come from increased levels of the activated, circulating T-cell pool in patients with AD.³⁶ Similarly, there are increased numbers of peripheral blood regulatory T cells in patients with AD.³⁷ Additional studies are needed to explain these contradictory results and solve the origin of iNKT cells.

We demonstrated that $V\alpha 24^+$ iNKT cells were more prevalent in human AD skin, especially in severe cases, although iNKT cells were not significantly increased in the skin of a murine AD model.³⁸ iNKT cells may play a distinct role in the skin of patients with AD because there was marked T-cell infiltration in AD skin lesions, which are histologically similar to allergic contact dermatitis, in which iNKT cells play a critical role in the mouse model.³⁹ The fact that iNKT cells are commonly present in T-cell infiltrates of lesional skin from allergic contact dermatitis patients⁴⁰ further supports our results (Fig 2, A). There are an estimated 2×10^{10} T cells in the skin of a normal individual, nearly twice the number of T cells in circulation, implying that the greater number of iNKT cells in AD skin than in PBMCs contributes to innate immunity in the pathogenesis of AD. Future investigations will focus on how skin iNKT cells increase along with conventional T-cell infiltration during AD.

We confirmed the previous finding that there is high TSLP expression mainly in the epidermal upper layer in AD skin, even as TSLP protein is barely detectable in sera of patients with AD.⁴¹ These observations imply that TSLP may exert its effects directly in inflammatory AD skin. This TSLP micromilieu in the skin of patients with AD partly contributes to the pathogenesis of nonallergic AD, unrelated to other allergic diseases, because high serum TSLP levels sufficient to sensitize the lung to allergic inflammation can trigger asthma as an atopic march in mouse AD models.⁴² We also confirmed that both TSLP receptor protein and mRNA are expressed on the surface of human iNKT cells, in agreement with the previous report on mouse iNKT cells.¹⁵ We hypothesize the existence of an innate immune axis, in which TSLP may activate innatelike iNKT cells in AD skin, to explain a new concept of the innate allergic immune response in the pathogenesis of AD.

Such functional studies that examine the effects of TSLP on iNKT cells are difficult to perform in patients with AD for ethical and safety reasons. We used blood samples from normal blood donors because the number of human iNKT cells is very small. Our findings that TSLP-treated iNKT cells secreted high amounts of IL-4 and IL-13, but not IFN- γ , compared with elevated expression of all 3 cytokines in α -GalCer-treated iNKT cells, imply that TSLP may directly activate iNKT cells to secrete T_H2 cytokines. These cells may contribute to innate allergic inflammation in the TSLP-rich environment of patients with AD. iNKT cells recognize lipids from plant pollens,^{43,44} suggesting that exogenous glycolipids from pollens entering the skin may directly activate iNKT cells and induce allergic skin inflammation. Our results are further supported by the observation that IL-7 and IL-18 also enhance IL-4

production by iNKT cells,^{45,46} and some cytokines such as IL-18 produced by LPS-stimulated DCs can activate iNKT cells without recognition of any antigen presented by CD1d.⁴⁷

We also found that iNKT cells cocultured with DCs in a TSLP-rich environment expressed more IFN- γ than TSLP-treated iNKT cells alone. This result implies that the interaction between DCs and iNKT cells in the presence of TSLP influenced iNKT cells to produce IFN- γ , as well as IL-4 and IL-13, in agreement with the previous finding that iNKT cells in AD skin expressed both IL-4 and IFN- γ .⁴⁸ Inflammatory dendritic epidermal cells (IDECs), a subset of DCs in AD, are considered important amplifiers of allergic inflammation in the skin. IDECs have a high stimulatory capacity toward T cells and might be involved in the switch from T_H2 -dominated acute AD to T_H1 -dominated chronic AD.⁴⁹ Further study is needed to determine whether IDECs may also cause iNKT cells to produce T_H1 cytokines in the chronic phase of AD, and to explain better how iNKT cells straddle the innate and adaptive allergic immune response in AD.

The adaptive allergic immune response, including IgE sensitization, has long been considered a key component of the allergic form of AD. The innate allergic immune response described here may explain the pathogenesis of nonallergic AD or steroid-recalcitrant severe chronic AD, because iNKT cells are resistant to the effects of corticosteroids and continuously produce cytokines at doses that inactivate T_H2 cells.⁵⁰ Our findings that expression of TSLP and iNKT cells in AD did not correlate with serum IgE levels (Fig E1), and the fact that iNKT cells, like mast cells, are so potent that even a small number of iNKT cells can produce robust cytokines independent of the adaptive immune response, further support this hypothesis.

Thymic stromal lymphopoietin-activated iNKT cells in AD skin may be an essential part of the innate allergic immune response in AD. Further research to identify specific glycolipid antigens that activate iNKT cells or new innate cells or substances in AD may help us better understand this type of allergic reaction.

We thank the patients and volunteers for their participation and cooperation.

Clinical implications: Invariant natural killer T cells, unlike conventional T cells, may contribute to the innate allergic immune response in the pathogenesis of atopic dermatitis.

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METHODS

ELISA for serum TSLP

Serum TSLP levels were determined by using commercially available ELISA kits (R&D Systems, Minneapolis, Minn) according to the manufacturer's instructions. Briefly, standards and samples were transferred to a microwell coated with anti-TSLP antibody at 100 μ L per well. The plates were incubated for 2 hours at room temperature. After washing with wash buffer 4 times, 100 μ L of the detection antibody was added to each well, and incubation was continued for 2 hours at room temperature. After another washing, streptavidin–horseradish peroxidase was added to the wells in the plate for detection of TSLP, and the plate was allowed to incubate for 20 minutes at room temperature. The color was developed by using substrate solution for 20 minutes at room temperature. Enzyme reactions were stopped with stop solution. Optical density was measured at 450 nm.

Immunofluorescence staining

Sorted 6B11⁺ iNKT cells treated with 1 or 50 ng/mL TSLP were stained with PE-antihuman IL-4 and IL-13 mAbs (BD Biosciences, San Jose, Calif). Cells were dried on a microscope slide, fixed with ethanol, dried again, embedded in liquid paraffin, and covered with a coverslip, and the edges were sealed. Acetone-fixed 6- μ m cryostat sections from lesional skin of patients with AD were stained with FITC-antihuman V α 24 (Beckman Coulter, Fullerton, Calif) and PE-antihuman IL-4 and IL-13 mAbs. Cell nuclei were stained with 4',6-diamidino-2-phenylindole. Fluorescent images of the iNKT cells in the double-stained (FITC/PE) whole mounts were obtained by using a confocal laser scanning microscope (LSM 510; Zeiss, Oberkochen, Germany).

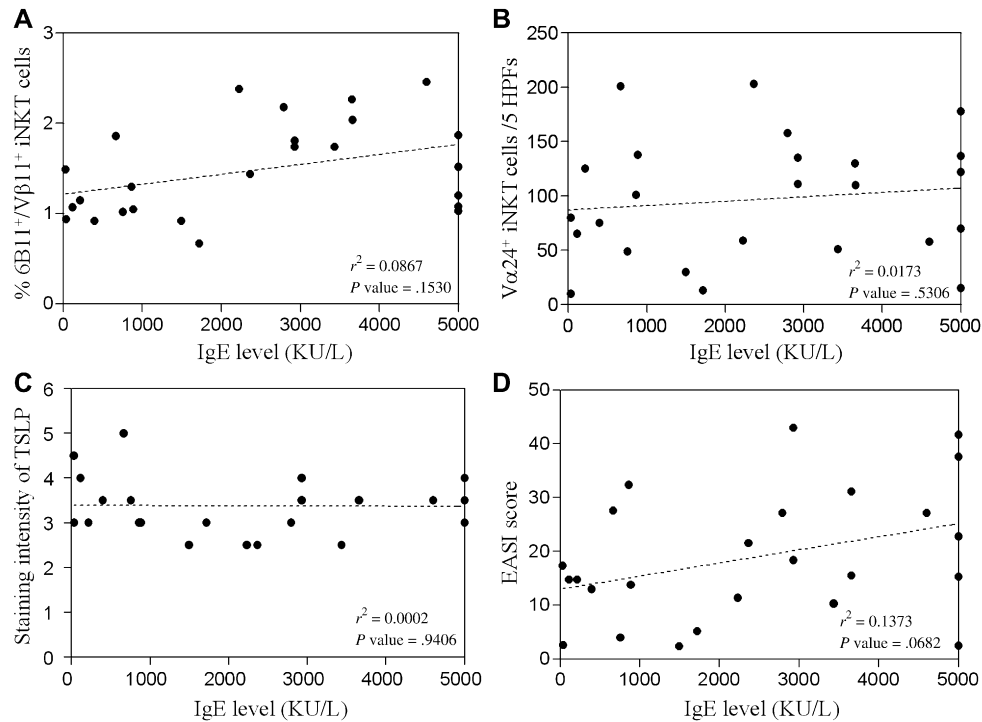


FIG E1. Serum IgE levels did not correlate with TSLP expression, iNKT cell levels, or the EASI scores of patients with AD. **A**, Correlation between serum IgE levels and the percentage of 6B11⁺/Vβ11⁺ iNKT cells in PBMCs of patients with AD (n = 25). Correlation between serum IgE levels and the number of Vα24⁺ iNKT cells (**B**) or the staining intensity of TSLP (**C**) in skin lesions of patients with AD (n = 25). **D**, Correlation between serum IgE levels and the EASI scores of patients with AD (n = 25).

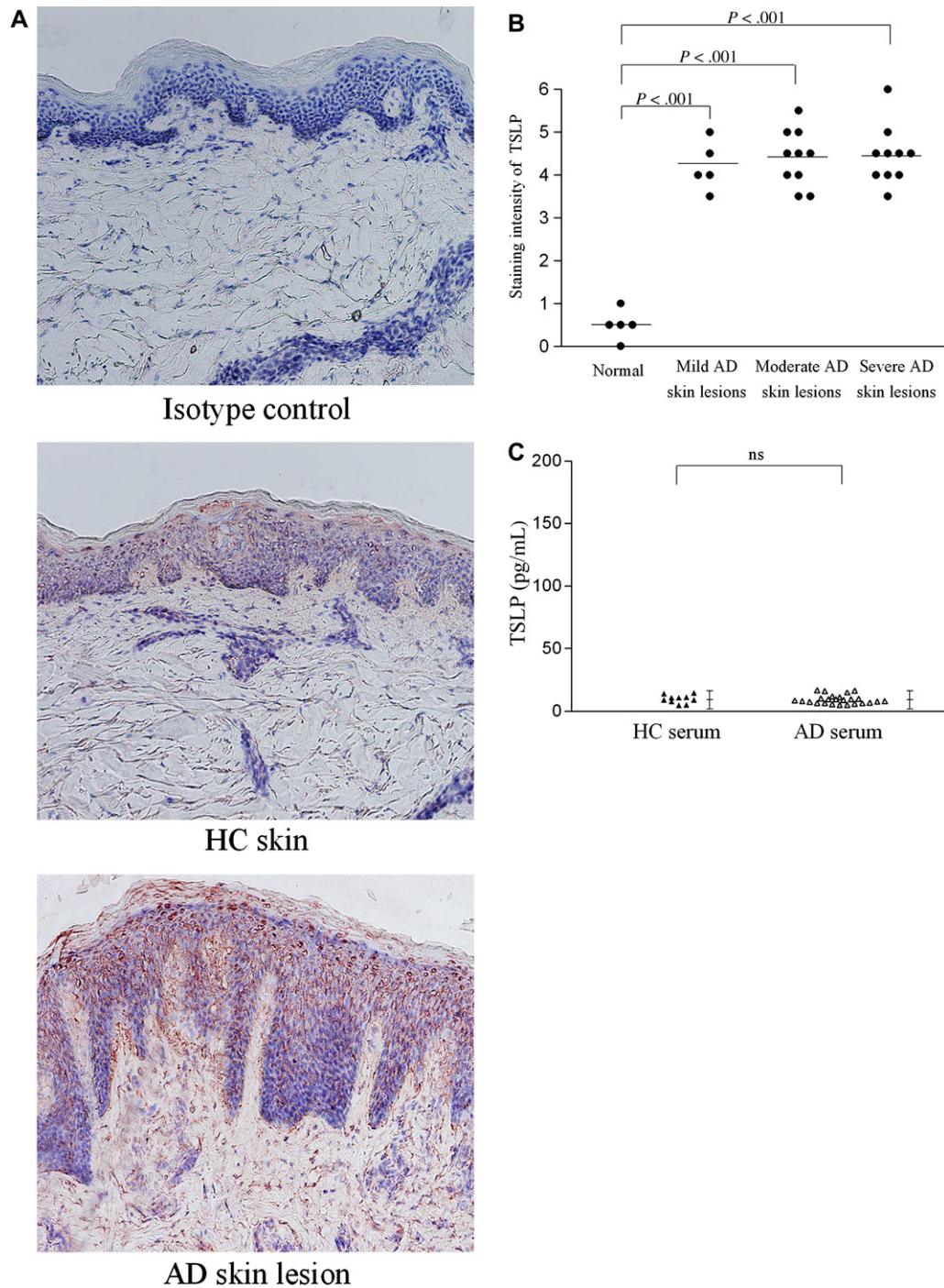


FIG E2. TSLP expression in lesional skin and sera of patients with AD. **A**, Representative immune-stained skin sections of HCs and patients with AD are shown. Original magnification $\times 100$. **B**, The intensity of TSLP staining was graded visually on a scale from 0 (no staining) to 6 (the most intense staining) for skin sections from HCs ($n = 5$) and mild ($n = 5$), moderate ($n = 10$), and severe AD skin lesions ($n = 10$). **C**, TSLP protein in the sera of HCs and patients with AD was evaluated by using ELISA. *ns*, Not significant.

TABLE E1. Characteristics of the study subjects*

Characteristics	Mild AD	Moderate AD	Severe AD
No. of patients (M:F)	5 (4:1)	10 (6:4)	10 (6:4)
Age (y)	22.2 ± 5.4	18.9 ± 3.8	24.3 ± 7.2
Total IgE (KU/L)	1801.7 ± 1705.1	1890.1 ± 1796.1	3287.2 ± 1654.9
Eosinophil (%)	3.84 ± 2.3	7.11 ± 4.3	9.79 ± 4.1†
EASI score	3.3 ± 1.1	14.4 ± 2.4	31.2 ± 7.4

*Age, total IgE, eosinophil, and EASI score are presented as mean ± SD.

†Statistically significant in comparison with mild AD ($P < .05$).