Christoph Plass, PhD[e]
Carl Hermann, PhD[b,i]
Gabriele I. Stangl, PhD[c]*
Roland Eils, PhD[b,d,i,j]*
Irina Lehmann, PhD[a,g]*

From [a]the Department of Environmental Immunology, Helmholtz Centre for Environmental Research—UFZ, Leipzig, Germany; [b]the Division of Theoretical Bioinformatics, German Cancer Research Center (DKFZ), Heidelberg, Germany; [c]the Institute for Agricultural and Nutritional Sciences, Martin Luther University Halle-Wittenberg, Halle, Germany; [d]Heidelberg Center for Personalized Oncology, DKFZ-HIPO, German Cancer Research Center (DKFZ), Heidelberg, Germany; [e]the Division of Epigenomics and Cancer Risk Factors, German Cancer Research Center (DKFZ), Heidelberg, Germany; [f]Research Group Genome Organization & Function, German Cancer Research Center (DKFZ) and Bioquant, Heidelberg, Germany; [g]Core Facility Studies, Helmholtz Centre for Environmental Research—UFZ, Leipzig, Germany; [h]Children's Hospital, Municipal Hospital "St Georg," Leipzig, Germany; [i]the Institute of Pharmacy and Molecular Biotechnology, and Bioquant Center, University of Heidelberg, Heidelberg, Germany; and [j]the Translational Lung Research Center Heidelberg, German Center for Lung Research (DZL), University of Heidelberg, Heidelberg, Germany. E-mail: irina.lehmann@ufz.de.

*These authors contributed equally to this study.

This work was supported by Helmholtz institutional funding (Helmholtz Centre for Environmental Research [UFZ] and German Cancer Research Centre—[DKFZ]) and funding by the Institute of Agricultural and Nutrition Science, Martin Luther University Halle-Wittenberg. Further support came from the German Cancer Research Center–Heidelberg Center for Personalized Oncology (DKFZ-HIPO).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

REFERENCES

1. Ramagopalan SV, Heger A, Berlanga AJ, Maugeri NJ, Lincoln MR, Burrell A, et al. A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. Genome Res 2010;20:1352-60.
2. Fetahu IS, Hobaus J, Kallay E. Vitamin D and the epigenome. Front Physiol 2014;5:164.
3. Herberth G, Hinz D, Roder S, Schlink U, Diez U, Borte M, et al. Innate versus adaptive immune response in newborns and their mothers: results from the LINA birth cohort study. Allergy 2010;65:645-6.
4. Weisse K, Winkler S, Hirche F, Herberth G, Hinz D, Bauer M, et al. Maternal and newborn vitamin D status and its impact on food allergy development in the German LINA cohort study. Allergy 2013;68:220-8.
5. Miyake Y, Tanaka K, Okubo H, Sasaki S, Arakawa M. Maternal consumption of dairy products, calcium, and vitamin D during pregnancy and infantile allergic disorders. Ann Allergy Asthma Immunol 2014;113:82-7.
6. Liu YJ, Soumelis V, Watanabe N, Ito T, Wang YH, Malefyt RD, et al. TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. Annu Rev Immunol 2007;25:193-219.
7. Takai T. TSLP expression: cellular sources, triggers, and regulatory mechanisms. Allergol Int 2012;61:3-17.
8. Ying S, O'Connor B, Ratoff J, Meng Q, Mallett K, Cousins D, et al. Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of TH2-attracting chemokines and disease severity. J Immunol 2005;174:8183-90.
9. Gauvreau GM, O'Byrne PM, Boulet LP, Wang Y, Cockcroft D, Bigler J, et al. Effects of an anti-TSLP antibody on allergen-induced asthmatic responses. N Engl J Med 2014;370:2102-10.

Available online August 15, 2015.
http://dx.doi.org/10.1016/j.jaci.2015.06.040

Effects of lidocaine on regulatory T cells in atopic dermatitis

To the Editor:

Atopic dermatitis (AD) is a chronic and relapsing skin disease characterized by inflammation and pruritus. In our previous hospital-based study of 1008 patients with AD, we found that the proportion of patients with severe AD was 10.7%.[1] Moreover, AD is a huge economic burden for families and society in general due to the long course of relapsed disease.

Regulatory T (Treg) cells control immune homeostasis and balance immune responses during inflammation. Treg cells suppress immune responses by interacting with effector T cells or antigen-presenting cells.[2] Recent clinical research has found that parents, particularly mothers, who have a history of atopy could have babies with less stable $FOXP3^+$ Treg cells in cord blood. These babies have the onset of AD less than 1 year after birth.[3] Therefore, it is likely that the abnormal numbers of Treg cells weaken the inhibition of T_H2 lymphocytes, thus resulting in AD inflammation.

Lidocaine is a widely used short-acting local anesthetic and antiarrhythmic agent. Previous studies demonstrated that lidocaine attenuated bronchoconstriction in patients with severe asthma, which enabled the dosage of oral corticosteroids to be reduced or eliminated in long-term treatment.[4] Because of the similarity of allergic diseases, lidocaine was used as a treatment for AD in China. Previous studies have shown that lidocaine dose-dependently inhibits the proliferative response and release of inflammatory factors from Staphylococcal enterotoxins A- and Staphylococcal enterotoxins B–stimulated PBMCs in patients with AD, contributing to clinical remission.[5]

In vivo, we sought to explore the effect of lidocaine on Treg cells and other key cytokines in patients with AD and murine AD models. Twenty patients were administered lidocaine (3 mg/kg per day) via a slow intravenous drip for 14 days. During

FIG 1. TSLP DMR, histone marks, and mRNA expression. **A,** Location of the *TSLP* DMR in relation to the *TSLP* gene *(upper part)*. The *TSLP* DMR shows a predicted chromosomal interaction with the *TSLP* promoter *(red bar)*. Furthermore, chromatin segmentation tracks generated by ChromHMM are displayed for 2 (high cord blood 25[OH]D_3) vs 2 (low cord blood 25[OH]D_3) samples over the region relating to the DMR and *TSLP* gene *(upper part)*, and a close-up of the DMR region *(lower part)*: Repressed chromatin states are observed in samples of low 25(OH)D_3 *(blue bars)* over the DMR region, and an ECNODE-predicted weak enhancer is found nearby (WE; *black bar*, *lower part* of the Fig 1, *A*). **B,** Association between *TSLP* enhancer methylation at birth (mean of 2 CpGs at Chr5:110.292.389-392, which marks the start of the enhancer region close to the *TSLP* DMR; analyzed via MassARRAY) and cord blood 25(OH)D_3; shown for all participants *(left column)*, only for those within the high vitamin D group (25[OH]D_3 >75th percentile, *middle column*), or for those within the low vitamin D group (25[OH]D_3 <25th percentile, *right column*). **C,** Association between methylation of the *TSLP* enhancer region (2 CpGs at Chr5:110.292.389-392) and *TSLP* mRNA expression, both analyzed in cord blood. **D,** Association between cord blood *TSLP* mRNA expression and 25(OH)D_3 concentrations. **E,** Association between *TSLP* mRNA expression at year 1 (first year) and cord blood 25(OH)D_3 concentration. **F,** *TSLP* mRNA expression at year 1 (first year) in children with (yes) or without (no) wheezing symptoms ever within the third year of life (*P* values from Mann-Whitney *U* test).

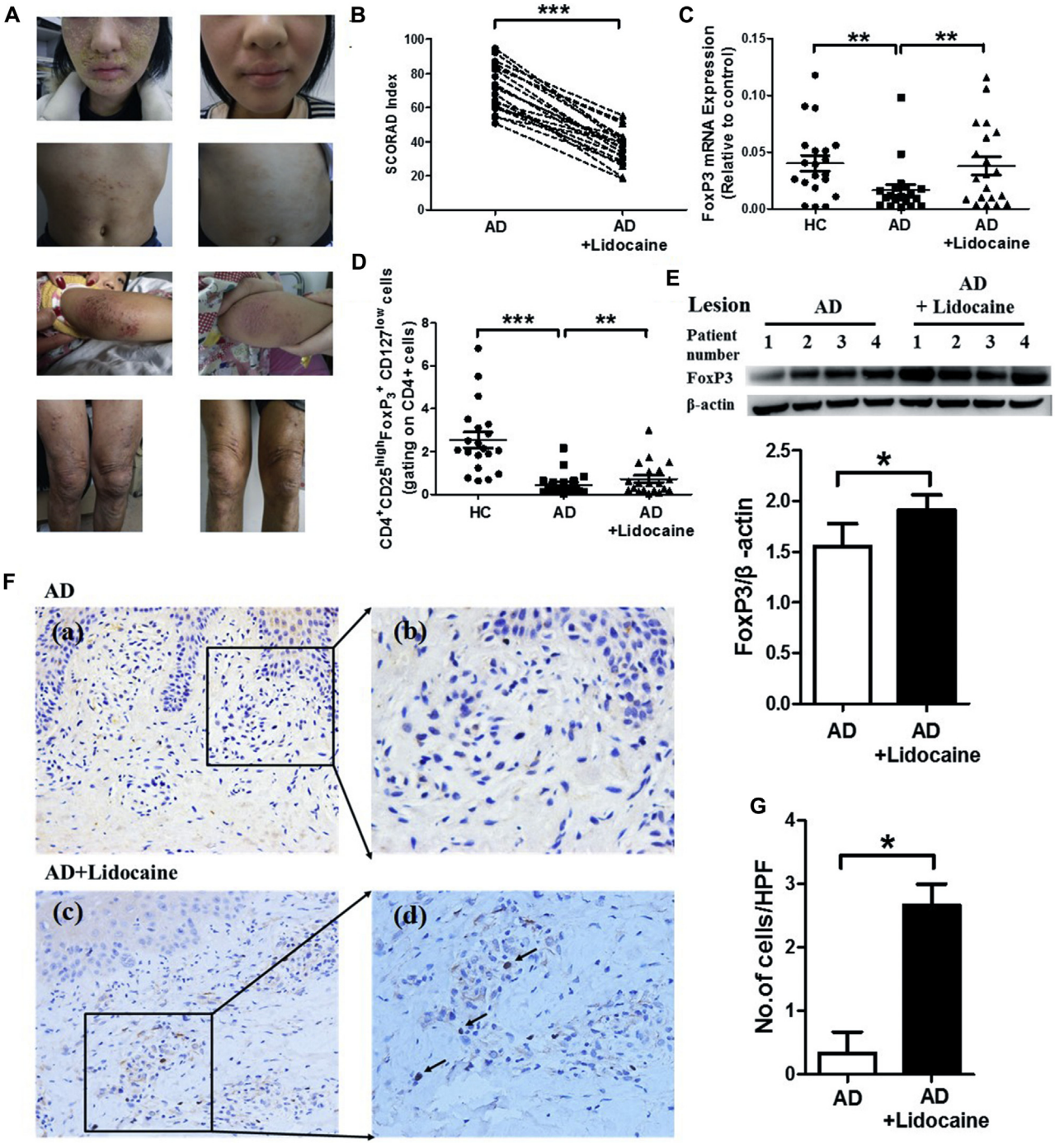

FIG 1. Effects of lidocaine therapy on lesion severity and Treg cells in patients with AD. **A,** Photographs of 4 patients with AD before and after lidocaine therapy (14 days). **B,** The SCORAD index of patients with AD declined significantly after lidocaine treatment (n = 20). **C** and **D**, *FOXP3* mRNA expression in PBMCs (via RT-PCR) and the proportion of Treg cells in PBMCs (via flow cytometric analysis) were upregulated in patients with AD after lidocaine treatment compared with before treatment (n = 20). **E,** FOXP3 protein expression in whole lesioned skin from patients with AD after lidocaine treatment was significantly increased compared with before treatment (n = 4) (via Western blot analysis). **F** and **G**, $FOXP3^{+}$ cell numbers in the lesioned skin increased in patients with AD after lidocaine therapy compared with before treatment (n = 3) (via immunochemistry analysis). Original magnification: *a* and *c*, ×100; *b* and *d*, ×200. **P* < .05, ***P* < .01, and ****P* < .001. *Bars* indicate mean ± SEM of 3 independent experiments. Statistical analyses were performed with paired *t* test. *HC*, Healthy control; *SCORAD*, SCORing Atopic Dermatitis.

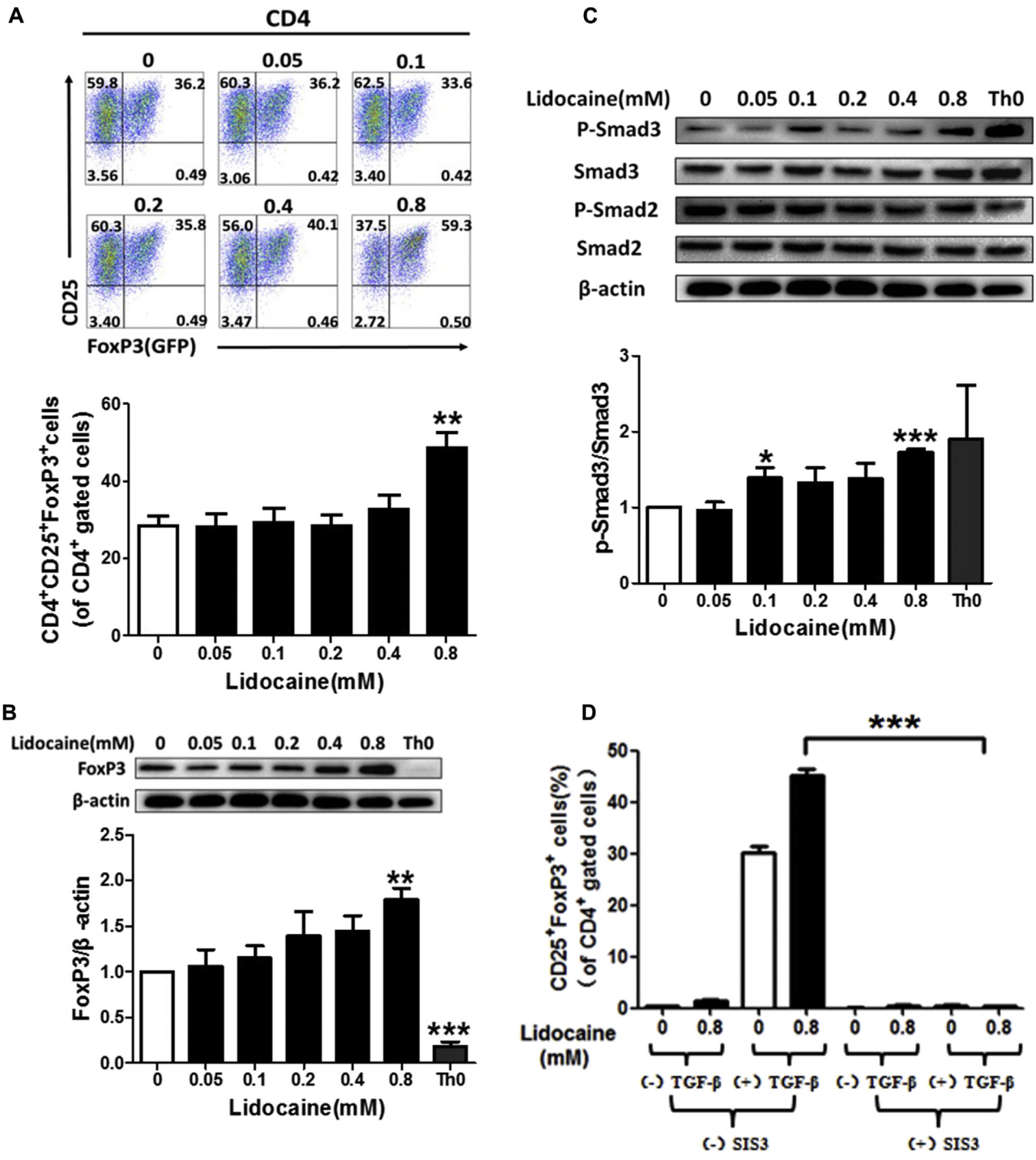

FIG 2. Effects of lidocaine on murine Treg-cell differentiation and its specific signaling pathway. **A-C,** *In vitro* Treg-cell differentiation: Lidocaine could upregulate the proportion of Treg cells (via flow cytometry), *FoxP3* protein expression level, and P-Smad3/Smad3 level (via Western blot) at a concentration of 0.8 mmol/L. **D,** SIS3 (5 μM) significantly blocked the promotion of lidocaine to Treg-cell differentiation at a concentration of 0.8 mmol/L (via Western blot). $*P < .05$, $**P < .01$, and $***P < .001$. *Bars* indicate mean ± SEM of 3 independent experiments. Statistical analyses were performed with the paired *t* test.

the treatment period, no adverse effects were observed in these patients. The clinical manifestations of AD markedly improved (Fig 1, *A*), and the SCORing Atopic Dermatitis index significantly declined (Fig 1, *B*). We found that *FOXP3* mRNA expression and the proportion of Treg cells in PBMCs from patients with AD were significantly decreased compared with that in healthy individuals. In addition, *FOXP3* mRNA expression level and the proportion of Treg cells in patients with AD after lidocaine treatment significantly increased compared with before treatment (Fig 1, *C* and *D*). Four patients permitted skin biopsies and their skin

sections were used to evaluate *FOXP3* mRNA and protein expression levels. Consistent with *FOXP3* mRNA expression levels in PBMCs, the expression of *FOXP3* mRNA and protein levels in lesioned skin was also upregulated (see Fig E1, *B*, in this article's Online Repository at www.jacionline.org; Fig 1, *E*). Immunochemistry analysis showed that $FOXP3^+$ cell numbers in the skin increased in the 3 patients with AD after lidocaine therapy (Fig 1, *F* and *G*). Furthermore, we established AD murine models with topical ovalbumin (OVA) as previously described,[6] of which each group included 5 mice. Then, we treated OVA-sensitized mice (via tail vein injection) with different doses of lidocaine: 1.5 mg/kg per day (OVA Lido1.5) and 3.0 mg/kg per day (OVA Lido3.0) for 7 days. A recent report has shown that cyclosporine elevates the proportion and absolute number of Treg cells in patients with AD; however, the function was not significantly different from that in healthy controls.[7] Therefore, cyclosporine (2.5 mg/kg per day) was intragastrically administered for 7 days as a positive control (OVA Cys). The phenotypes of mice treated with lidocaine and cyclosporine improved compared with mice treated with saline (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). In addition, the index of lesion severity and histopathological findings were consistent with the improved phenotypes (Fig E2, *B* and *C*). We subsequently measured *FoxP3* mRNA and protein expression in whole lesioned skin using RT-PCR and Western blot. We also used flow cytometric analysis to determine the proportion of Treg cells in the dermis of lesioned skin, spleen, and lymph nodes in the 4 treatment groups. Compared with the OVA saline group, the proportion of Treg cells of murine lesions and *FoxP3* mRNA and protein expression levels in the lidocaine treatment groups and the cyclosporine treatment group were significantly upregulated (see Fig E3, *A*-*C*, in this article's Online Repository at www.jacionline.org). However, no significant difference was detected in the proportion of Treg cells in murine spleen and lymph nodes from the OVA Lido1.5 and OVA Lido3.0 treatment groups and the OVA Cys treatment group (Fig E3, *E*). Furthermore, lidocaine therapy ameliorated the T_H1/T_H2 cell and IL-17A/IL-17E cytokine imbalance in patients with AD and murine AD models. In patients with AD, *IFN-γ*, *IL4*, and *IL17A* mRNA expression levels were significantly upregulated in PBMCs after lidocaine treatment in contrast to those before treatment while *IL17E* mRNA level was downregulated significantly (Fig E1, *A*). Furthermore, *IL17E* mRNA level of whole lesioned skin decreased significantly in patients with AD after lidocaine treatment (Fig E1, *B*). IL-17E is involved in the T_H2-dominant immune response and its downregulation might contribute to the amelioration of symptoms. Moreover, the relationship between IFN-γ and IL-4 as well as that between IL-17A and IL-17E is interacting. Consequently, we presumed that IL-4 mRNA expression was upregulated to inhibit the elevation in IFN-γ mRNA expression while IL-17A mRNA expression increased in response to the decrease in IL-17E mRNA expression level. Although lidocaine therapy did not totally block the upregulation of cytokines mentioned above, it still disrupted the amplification cycle of skin inflammation and ameliorated symptoms. Owning to the less complicated neuroendocrine-immune networks and habitation in pathogen-free environment, the regulation of inflammatory factors in murine models was more explicit. Compared with the OVA saline group, the expression of IFN-γ mRNA in the OVA Lido3.0 group was significantly upregulated, whereas IL-4 and IL-17E mRNA levels in the OVA Lido1.5, OVA Lido3.0, and OVA Cys groups were significantly downregulated (Fig E3, *D*).

In vitro, the splenocytes of FoxP3-EGFP mice were purified to prepare $CD4^+CD25^-$ cells as naive $CD4^+$ T cells. Cells were stimulated with antibodies against CD3 (10 μg/mL), CD28 (2 μg/mL), TGF-β (5 ng/mL), and IL-2 (40 ng/mL). The cells were collected for flow cytometry and Western blot analysis 4 days later. First, we used CCK8 analyses to identify safe levels of lidocaine and determined that 0.8 mM was appropriate (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org). At this concentration, lidocaine upregulated the proportion of Treg cells and FoxP3 protein expression level (Fig 2, *A* and *B*). Western blot analyses were used to elucidate potential target signaling pathways. We found that 0.8 mM lidocaine significantly promoted Treg-cell differentiation, activating the Smad3/TGF-β signaling pathway (Fig 2, *C*), but not through the Smad2/TGF-β or nuclear factor kappa B signaling pathways (Fig 2, *C*; Fig E4, *B*). SIS3 (5 μM), an inhibitor of Smad3, was used to define the authenticity of lidocaine-induced regulation of Treg cells. We found that SIS3 significantly downregulated the proportion of Treg cells in the presence of 0.8 mM lidocaine, which established categorically that Treg cells regulated by lidocaine were at least in part, if not wholly, dependent on Smad3 activity (Fig 2, *D*).

To our knowledge, the present study is the first report to demonstrate that lidocaine therapy can ameliorate the severity of lesioned skin in patients with AD and murine AD models. Our study has also uncovered the critical role and molecular mechanism of lidocaine in Treg-cell regulation. In addition, we found that lidocaine promoted *FoxP3* transcription by activating TGF-β–induced Smad3 phosphorylation. Lidocaine therapy also ameliorated the T_H1/T_H2 cell and IL-17A/IL-17E cytokine imbalance in patients with AD and murine AD models. These novel findings indicate that lidocaine might exert regulatory effects on immune cells and thus reduce AD inflammation.

Huaguo Li, MD[a]*
Chunxiao Li, MS[a]*
Hui Zhang, PhD[a]
Lingyun Zhang, PhD[b]
Ruhong Cheng, MD[a]
Ming Li, PhD[a]
Yifeng Guo, MD[a]
Zhen Zhang, MS[a]
Zhiyong Lu, PhD[a]
Yin Zhuang, MS[a]
Ming Yan, MS[a]
Yan Gu, MD[a]
Xiaobo Feng, MS[a]
Jianying Liang, MS[a]
Xia Yu, MS[a]
Honglin Wang, PhD[b]
Zhirong Yao, PhD[a]

From [a]the Department of Dermatology, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, and [b]Shanghai Institute of Immunology, Institute of Medical Sciences, Shanghai Jiao Tong University School of Medicine, Shanghai, China. E-mail: dermatology.yao@sohu.com. Or: honglin.wang@sjtu.edu.cn.

*These authors contributed equally to this work as first authors.

This study was supported by grants from the Public Welfare Research Fund for Healthcare (grant no. 201202013), 973 program (grant no. 2014CB541905), the National Nature Science Foundation of China (grant nos. 81171544, 81472897, 81201227, and 31330026), and the Science and Technology Commission of Shanghai Municipality (grant no. 13XD1402900).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

REFERENCES

1. Shi M, Zhang H, Chen X, Guo Y, Tao J, Qi H, et al. Clinical features of atopic dermatitis in a hospital-based setting in China. J Eur Acad Dermatol Venereol 2011;25:1206-12.
2. Pellerin L, Jenks JA, Bégin P, Bacchetta R, Nadeau KC. Regulatory T cells and their roles in immune dysregulation and allergy. Immunol Res 2014;58:358-68.
3. Hinz D, Bauer M, Roder S, Olek S, Huehn J, Sack U, et al. Cord blood Tregs with stable FOXP3 expression are influenced by prenatal environment and associated with atopic dermatitis at the age of one year. Allergy 2012;67:380-9.
4. Hunt LW, Frigas E, Butterfield JH, Kita H, Blomgren J, Dunnette SL, et al. Treatment of asthma with nebulized lidocaine: a randomized, placebo-controlled study. J Allergy Clin Immunol 2004;113:853-9.
5. Jiao Q, Wang H, Hu Z, Zhuang Y, Yang W, Li M, et al. Lidocaine inhibits staphylococcal enterotoxin-stimulated activation of peripheral blood mononuclear cells from patients with atopic dermatitis. Arch Dermatol Res 2013;305:629-36.
6. Spergel JM, Mizoguchi E, Brewer JP, Martin TR, Bhan AK, Geha RS. Epicutaneous sensitization with protein antigen induces localized allergic dermatitis and hyperresponsiveness to methacholine after single exposure to aerosolized antigen in mice. J Clin Invest 1998;101:1614-22.
7. Brandt C, Pavlovic V, Radbruch A, Worm M, Baumgrass R. Low-dose cyclosporine A therapy increases the regulatory T cell population in patients with atopic dermatitis. Allergy 2009;64:1588-96.

Available online September 12, 2015.
http://dx.doi.org/10.1016/j.jaci.2015.07.039

Decreased IgM, IgA, and IgG response to pneumococcal vaccine in children with transient hypogammaglobulinemia of infancy

To the Editor:

Transient hypogammaglobulinemia of infancy (THI) is a primary antibody deficiency occurring in the first years of life and is characterized by a delay in the immunoglobulin production that spontaneously recovers in early infancy. In the young symptomatic child with low IgG levels and more than 2% B cells, there are no peculiar clinical and immunologic features that allow discrimination between self-limiting THI, common variable immunodeficiency, or other dysgammaglobulinemias. Only those patients whose IgG levels have normalized after age 4 years have a definitive THI diagnosis made *a posteriori*.[1,2]

In a previous study, we demonstrated that a subgroup of children with putative THI shares abnormalities of B-cell memory subsets with other defined immune deficiencies, and cautiously suggested the term "hypogammaglobulinemia during infancy" to define conditions in which it is not yet possible to discriminate THI from other primary antibody defects.[3] Recently, according to the new European Society for Immunodeficiencies diagnostic criteria, a working definition of "unclassified hypogammaglobulinemia" (UH) has been introduced to register patients with IgG values below age-related normal values detected in the first 3 years of life that will be moved to THI diagnosis if there is spontaneous resolution before age 4 years (http://esid.org/Working-Parties/Registry/Diagnosis-criteria).

Measurement of pneumococcal antibodies is an important tool in the immunologic assessment of patients with suspected immune deficiencies.[4]

The aim of this study was to analyze prospectively IgM-, IgA-, and IgG-antibody responses to pneumococcus vaccine (PV) in children with UH until THI or other immune deficiencies criteria were met.

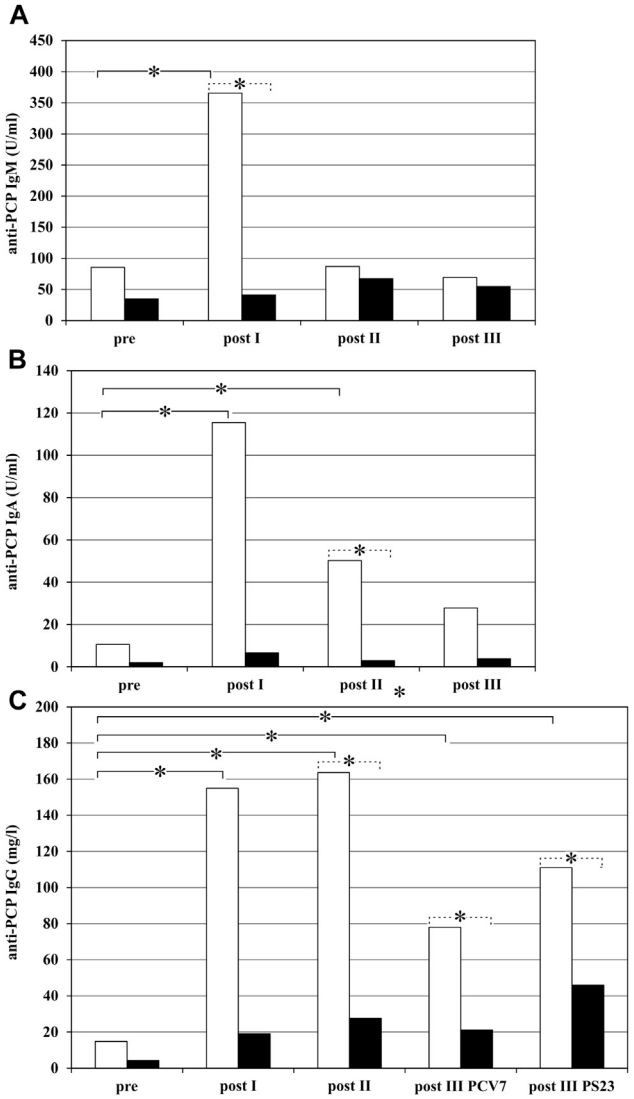

FIG 1. Median IgM **(A)**, IgA **(B)**, and IgG **(C)** anti-PCP antibodies in HC *(white bars)* and children with UH *(black bars)* before (pre) and 4 weeks after each PV dose (post-I, post-II, and post-III). **P* values <.05. The *continuous line* compares pre- and postvaccination titers within the same cohort. The *dotted line* compares postvaccination titers between HC and children with UH.

In the context of the Italian Primary Immunodeficiency Network, we enrolled 21 patients (age range, 12-36 months) with a history of recurrent infections who had an initial diagnosis of UH in accordance with our national protocol for THI (http://www.aieop.org/stdoc/prot/rec_thi_en_06.pdf) and monitored them until age 4 years with regular clinical and immunologic evaluations. Eighteen healthy age-matched children (HC) (age range, 15-40 months) were used as controls. Informed consent was obtained from the patient's parents. The Institutional Ethical Committee approved the study. All patients and HC received 3 doses of 7-valent conjugated pneumococcus vaccine (PCV 7, Prevnar, Pfizer), with the exception of 5 children with UH and 6 HC who received the third PV dose with unconjugated pneumococcus polyvalent vaccine (Pneumo23; Aventis, Milan, Italy) because they were older than 2 years. Serum samples were collected from children with UH and HC before and 4 weeks after each

METHODS

Subjects

Patients. Before the study, patient consent and ethical approval were obtained. Subjects included in this study were 20 patients with AD with lidocaine therapy and 20 healthy age- and sex-matched volunteers. Patients with AD younger than 65 years were severely affected and fulfilled the criteria of Hanifin and Rajka. The clinical severity during therapy was monitored by using the SCORing Atopic Dermatitis index (0-24, mild AD; 25-50, moderate AD; 51-103, severe AD). None of the patients had other skin diseases besides AD, and they did not receive any systemic corticosteroids in the recent 3 months.

Mice. Balb/C mice (6- to 8-week-old female mice) were purchased from Slack Laboratory (Shanghai, China). The mice were anesthetized and their backs were shaved. Skin injury was induced by repeated tape-stripping, after which 100 mg of OVA in 100 mL of saline, or saline only, was applied on a specific patch and attached to the injured area. Treatment was repeated with the 1-week sensitization period after which a 2-week resting period was kept before the second and similar sensitization week. The lesional severity was assessed every week. The standards used are shown as follows: 0 point for none, 1 point for mild, 2 points for moderate, and 3 points for severe lesioned skin. The symptoms included erythema, papule/edema, excoriation, oozing, lichenification, and dry skins. At the end of the seventh week, the mice in the saline group and the OVA group were sacrificed and samples were collected 24 hours after the last treatment. The other 4 groups were treated with saline, lidocaine 1.5 mg/kg per day and lidocaine 3.0 mg/kg per day with tail vein injection, and cyclosporin 2.5 mg/kg per day by intragastric administration for 7 days, which were accompanied by sustained OVA sensitization (each group n = 5). Mice in the above 4 groups were sacrificed at the eighth week.

FoxP3-EGFP mice were offered by the research group of Professor Honglin Wang in the Institute of Medical Sciences, Shanghai Jiao Tong University School of Medicine.

Naive $CD4^+$ T cells were prepared with FoxP3-EGFP mice.

Cells. PBMCs were isolated from heparinized venous blood from patients with AD with Ficoll-Paque plus (Pharmacia, Uppsala, Sweden) within 6 hours after bleeding by means of density gradient centrifugation and cells were resuspended in RPMI 1640 medium supplemented with 5% pooled type AB normal human serum (Sigma-Aldrich, St Louis, Mo) for further total RNA extraction and flow cytometry.

Murine $CD4^+$ T cells were purified from murine splenocytes by negative selection, which used an MACS system with biotin-antibody cocktail and antibiotin microbeads. Negatively selected $CD4^+$ cells were further positively selected into $CD4^+CD25^-$ cells as naive $CD4^+$ T cells by using $CD25^-$ PE antibody and anti-PE MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany).

Single-cell suspensions from the murine inflamed skins were prepared by cutting the skin samples into small pieces, then passing them through a 70-μm cell strainer, washing with PBS, and filtrating with a 40-μm cell strainer again (Becton-Dickinson & Company, Franklin Lakes, NJ).

For the preparation of dermal single-cell suspension, lesioned skins were cut into 3 mm × 3 mm sections and immersed in DispaseII at 4°C overnight. The sections were then washed thrice by Hank's fluid without Ca^{2+} and Mg^{2+}. The dermis was gently isolated with ophthalmic forceps and vibrated with collagenase at 37°C for 20 minutes and with DNAase for the next 25 minutes. (All the products mentioned above were from Sigma-Aldrich.)

Analysis of lidocaine cytotoxicity on PBMCs

$CD4^+$ T cells (1×10^5 for cells/well) were seeded onto a 96-well plate in medium with FBS. Lidocaine of different concentrations was added into the plate (0, 0.1, 0.2, 0.4, 0.8, 1.2, 1.6, and 2.0 mM). Water-soluble tetrazolium salts were added to each well (CCK-8, Dojindo, Mashiki-machi, Kamimashiki-gun, Kumamoto, Japan). Cells were incubated for 4 hours at 37°C. Microplate reader was used to detect absorbance, which was measured at 450 nm with a reference filter at 650 nm.

In vitro Treg-cell differentiation

Naive $CD4^+$ T Cells were cultured in complete medium. Purified $CD4^+CD25^-$ cells were stimulated with antibodies against CD3 (10 μg/mL) and CD28 (2 μg/mL) (Becton-Dickinson & Company) and TGF-β (5 ng/mL) and IL-2 (40 ng/mL) (R&D Systems, Minneapolis, Minn). SIS3 (5 μM) was added to the culture at 18 hours after iTreg-cell differentiation to define the specificity of lidocaine-mediated Smad3 induction.

Flow cytometry

Anti-mouse CD4 APC and anti-mouse CD25 PerCP-Cyanine5.5 were used for surface staining. Intracellular staining for FoxP3 and Helios was done with anti-mouse/rat FoxP3 PE and anti-Helios FITC according to the manufacturer's instruction (eBioscience, San Diego, Calif).

Isolated 1×10^6/mL PBMCs were stained with human Treg-cell staining kit. Briefly, cells were suspended in a staining buffer and incubated with a surface marker (CD4, CD25, CD127) for 30 minutes at 4°C in the dark. After 6 minutes of washing with the staining buffer at 300 g, cells were incubated overnight with fixation/permeabilization buffer at 4°C in the dark. Subsequently, the cells were stained with PE anti-human FOXP3 for 30 minutes at room temperature in the dark and washed twice (eBioscience).

RNA isolation and analysis

Total RNA was extracted from PBMCs and murine skins with TRIzol Reagent (Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions. It was used as a template for cDNA synthesis by using SuperScript III reverse transcriptase (Invitrogen). Real-time quantitative PCR analysis was performed with 7500 Fast Real Time PCR system (Applied Biosystems, Foster City, Calif) using primers and probes. β-Actin was used as an endogenous control. All primers and probes were purchased from Sangon Biotech (Shanghai, China).

Western blot analysis

Western blot analysis was performed with anti-FoxP3 (eBioscience), Smad3, Phospho-Smad3, Smad2, Phospho-Smad2, nuclear factor kappa b p65, Phospho-nuclear factor kappa b p65, IκBα, Phospho-IκBα rabbit mAb, and anti–β-actin mouse mAbs (Cell Signaling Technology, Beverly, Mass) followed by horseradish peroxidase–conjugated goat anti-rabbit IgG and goat anti-mouse IgG (H+L) (Beyotime Biological Technology, Jiangsu, China) and ECL detection (Thermo Fisher Scientific, Inc, Waltham, Mass).

Histology

Skins were fixed in 10% formalin and embedded in paraffin. Multiple 4-μm-thick sections were stained with hematoxylin-eosin.

Immunochemistry

Samples obtained from exposed skin were immersed in 4% formamint-fixed, and paraffin-embedded blocks were archived. Sections (4 μm) were used for immunoperoxidase staining to detect FOXP3 (eBiosciences). Horseradish peroxidase-conjugated secondary antibody anti-rat IgG (H+L) was purchased from Sangon Biotech.

Statistical analyses

Data were analyzed by using the GraphPad Prism 5 package (GraphPad Inc, La Jolla, Calif). Two-tailed student *t* tests and nonparametric Mann-Whitney test were used to compare values. P values are designated as $P < .05$ (*), $P < .01$ (**), and $P < .001$ (***).

Study approval

All participants or their legal guardians provided informed consent. All procedures, forms, and protocols were approved by the Ethics Committee of Xinhua Hospital, affiliated with Shanghai Jiao Tong University School of Medicine. The Animal Care and Use Committee of Xinhua Hospital approved all animal experiments.

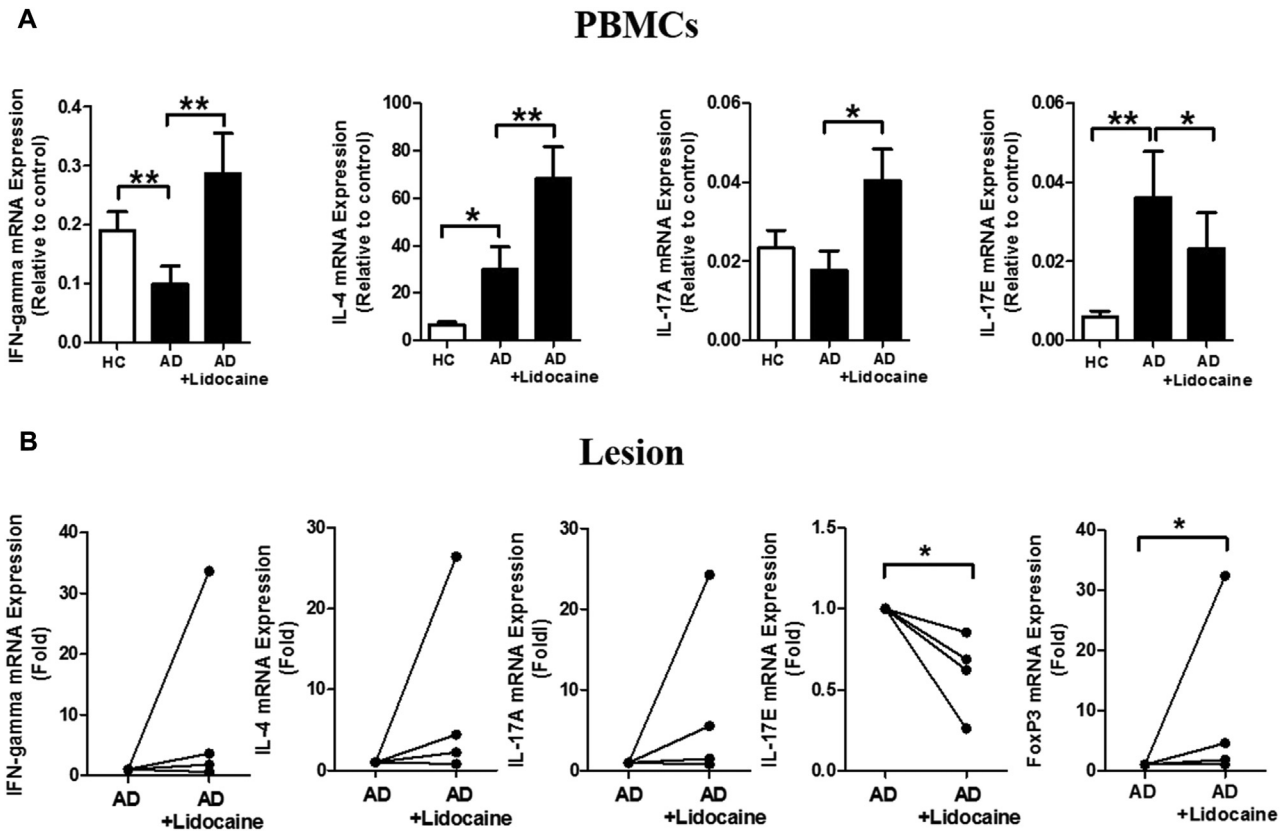

FIG E1. Lidocaine regulated other cytokines in PBMCs and lesioned skins of patients with AD. **A,** IFN-γ, IL-4, and IL-17A mRNA expression levels were significantly upregulated in PBMCs of patients with AD after lidocaine treatment in contrast to those before treatment, whereas IL-17E mRNA level was downregulated significantly (n = 20). **B,** *FOXP3* mRNA level significantly increased, whereas IL-17E mRNA level decreased in whole lesioned skins of patients with AD after lidocaine treatment in contrast to those before treatment (n = 4). **P* < .05 and ***P* < .01. *Bars* indicate mean ± SEM of 3 independent experiments. Statistical analyses were performed with nonparametric Mann-Whitney test. *HC*, Healthy control.

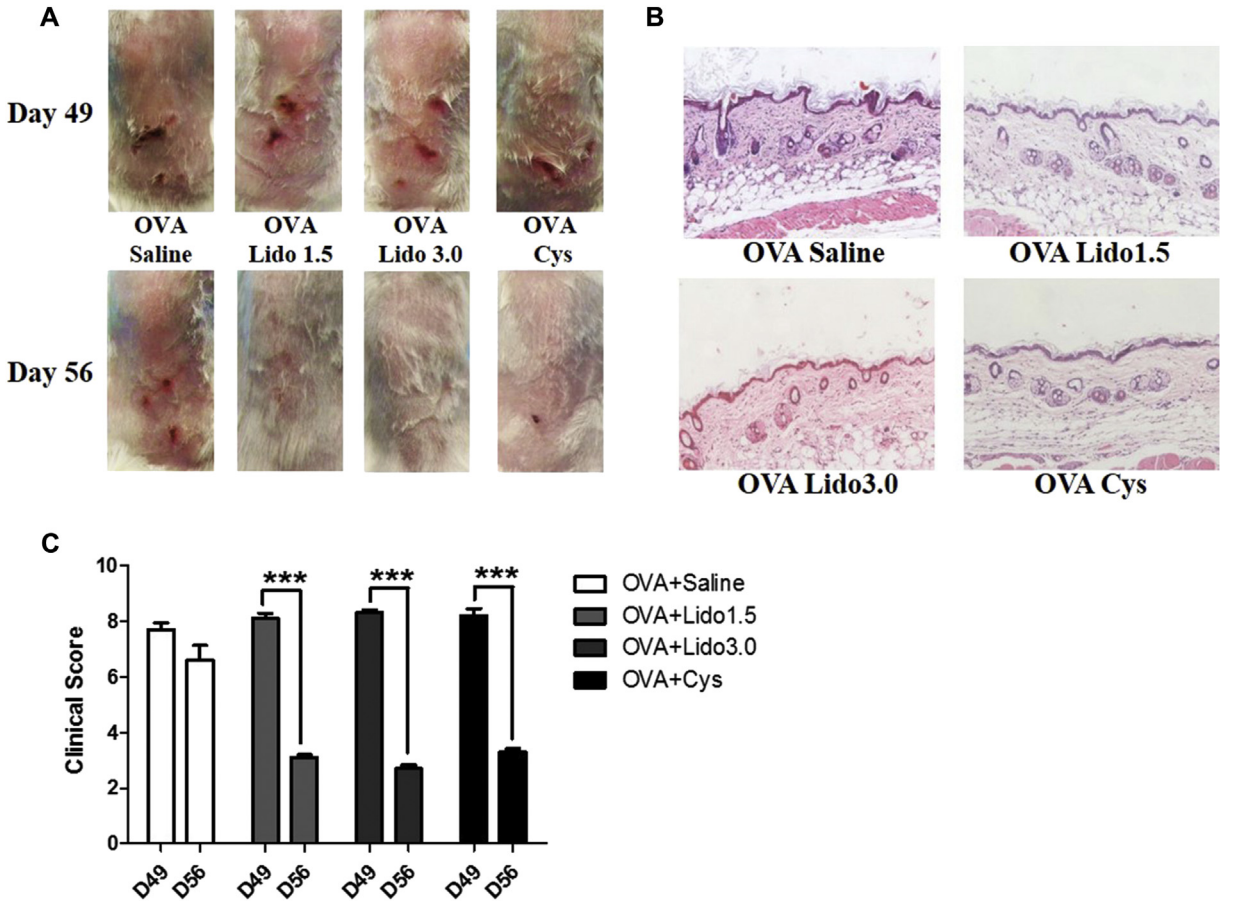

FIG E2. Effects of lidocaine therapy on lesional severity in OVA-sensitized murine models. **A,** Lidocaine significantly improved the phenotypes in mice of lidocaine (OVA Lido1.5 and OVA Lido3.0) and cyclosporin (OVA Cys) treatment groups compared with the OVA saline group (n = 5). **B,** Lidocaine significantly improved the histopathologic findings with significant decline in the number of infiltrating cells in mice of lidocaine (OVA Lido1.5 and OVA Lido3.0) and cyclosporin (OVA Cys) treatment groups compared with the OVA saline group (n = 5). Original magnification: ×50. **C,** Except the OVA saline group, the murine scores of severity in the other 3 groups (OVA Lido1.5, OVA Lido3.0, ad OVA Cys) after treatment were significantly decreased in contrast to those before treatment (n = 5). *Bars* indicate mean ± SEM (n = 5) of 2 independent experiments. Statistical analyses were performed with the paired *t* test. ***$P < .001$.

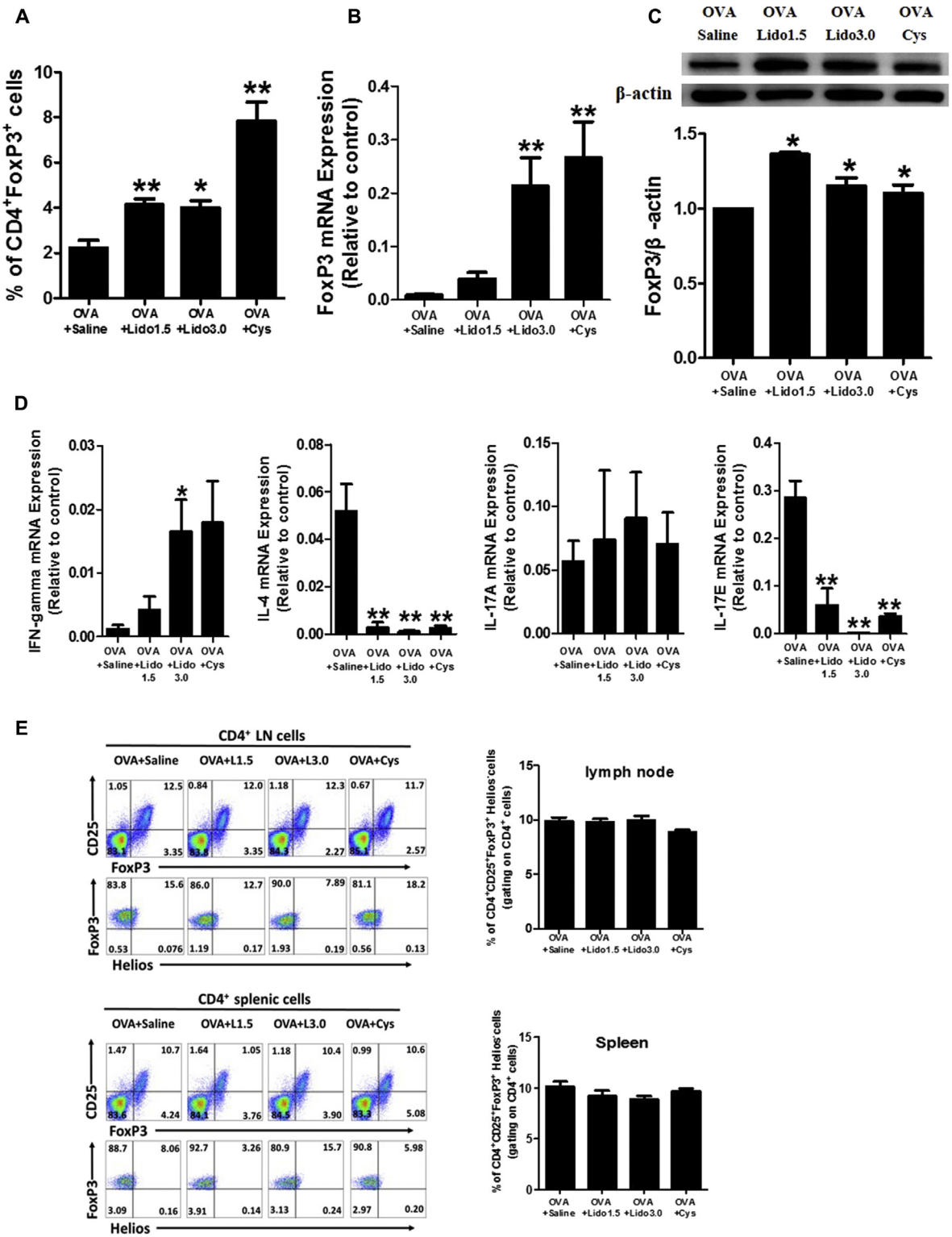

FIG E3. Effects of lidocaine therapy on OVA-sensitized murine models. **A-C,** Compared with those of the OVA saline group, the proportion of Treg cells (via flow cytometry) in murine dermis of lesioned skin and *FoxP3* mRNA (via RT-PCR) and protein expression level (via Western blot) of whole lesioned skins in the lidocaine treatment group and the cyclosporin treatment group (OVA Cys) were significantly upregulated. **D,** Compared with those of the OVA saline group, murine IFN-γ mRNA expression level of the OVA Lido3.0 group was significantly upregulated while IL-4 and IL-17E mRNA expression levels of lidocaine treatment groups (OVA Lido1.5 and OVA Lido3.0) and the cyclosporin treatment group (OVA Cys) were significantly downregulated. No significant difference in IL-17A mRNA expression level was detected in any groups. **E,** No significant difference was detected in the proportion of Treg cells in murine spleen and lymph nodes in lidocaine treatment groups (OVA Lido1.5 and OVA Lido3.0) and the cyclosporin treatment group (OVA Cys). $*P < .05$ and $**P < .01$. *Bars* indicate mean ± SEM (n = 5) of 2 independent experiments. Statistical analyses were performed with nonparametric Mann-Whitney test.

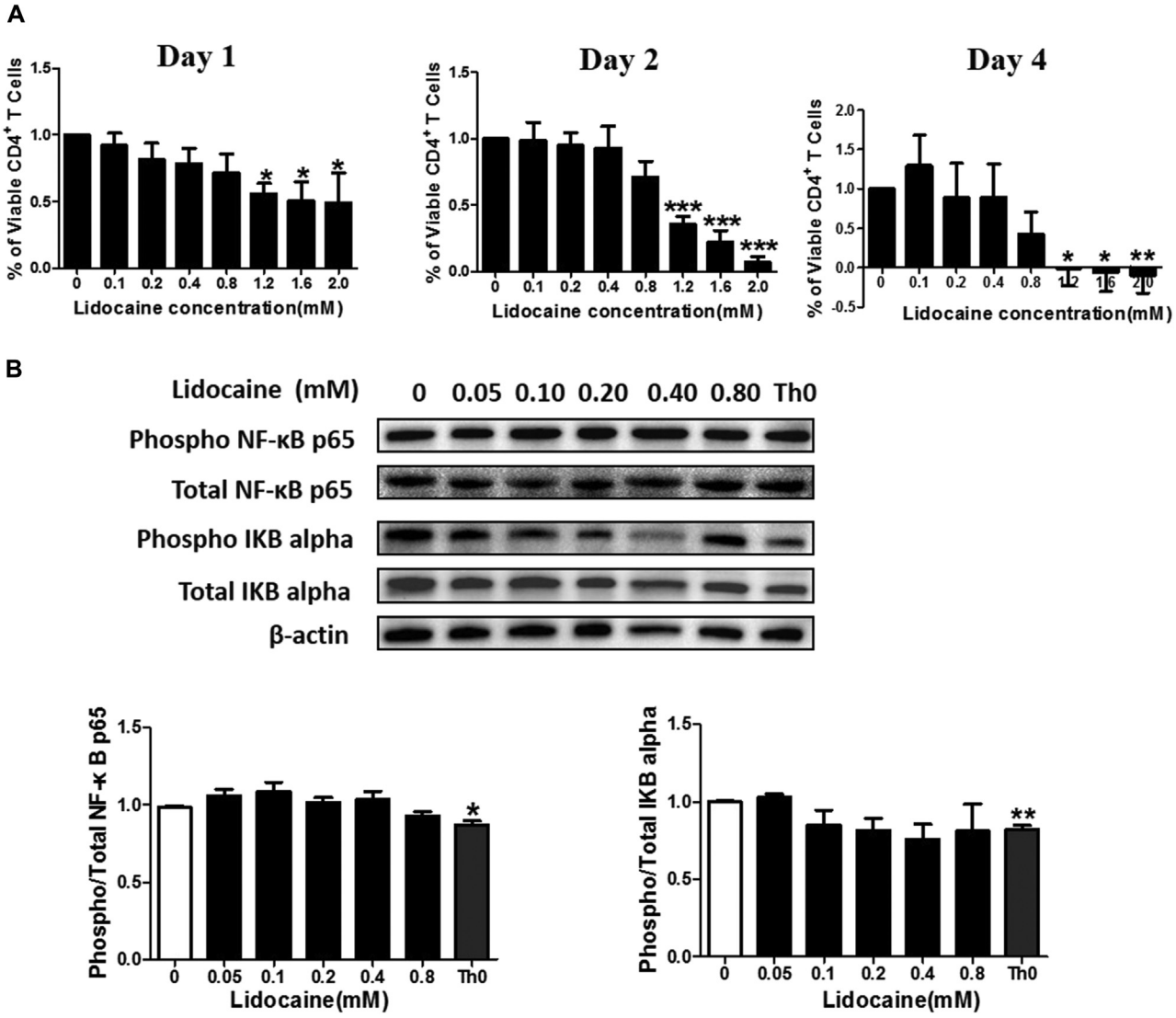

FIG E4. Effects of lidocaine on murine Treg-cell differentiation and its specific signaling pathway. **A,** The safety level of lidocaine was verified as 0 to 0.8 mmol/L by CCK8 analysis. **B,** At the concentration of 0.8 mmol/L, the promotion of lidocaine to Treg-cell differentiation was not significantly relevant with nuclear factor kappa B signaling pathway (via Western blot). $*P < .05$, $**P < .01$, and $***P < .001$. *Bars* indicate mean ± SEM of 3 independent experiments. Statistical analyses were performed with paired *t* test.