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Research paper

FABP5 as a possible biomarker in atopic march: FABP5-induced Th17 polarization, both in mouse model and human samples

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ABSTRACT

Background: While the incidence of patients with atopic dermatitis (AD) with atopic march (AM) showing respiratory allergy is steadily rising, the pathomechanism is still unknown. There are currently no biomarkers to predict progression of AM.

Methods: To explore the mechanism of AM, patients with AD and AM and healthy controls were recruited and RNA microarray, flow cytometry, quantitative real-time polymerase chain reaction, and immunofluorescence staining were performed. We also co-cultured dendritic cells and $CD4^+$ *T* cells with various *Dermatophagoides farinae* allergen fractions. Cytokine levels were evaluated using enzyme-linked immunosorbent assay.

Findings: Both fatty-acid-binding protein 5 (FABP5) and Th17-related genes were more highly expressed in AM. FABP5 knockdown significantly decreased Th17-inducing cytokines in keratinocytes and IL-17A in T cells from AM patients. Further confirmation was obtained using an AM mice model compared to mice without AM. Der f 1, a major *D. farinae* allergen, increased FABP5 and IL-17A expression in T cells from AM patients. Higher serum FABP5 levels from AM patients were positively correlated with serum IL-17A levels.

Interpretation: FABP5 expression, possibly enhanced by higher epicutaneous and respiratory sensitization to Der f 1, may directly promote Th17 responses in AD patients with AM. Thus, AM progression can be explained by Th17 reaction induced by FABP5. FABP5 was identified as a potential biomarker in AM.

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Abbreviations: AA, Allergic asthma; AD, Atopic dermatitis; AHR, Airway hyperresponsiveness; AM, Atopic march; AR, Allergic rhinitis; D. farinae, *Dermatophagoides farinae*; Der f 1, *Dermatophagoides farinae 1*; EASI, Eczema area and severity index; E-FABP, Epidermal fatty acid-binding protein; ELISA, Enzyme-linked immunosorbent assay; FABP, Fatty acid-binding protein; FABP5L, Fatty acid-binding protein5-like; HDM, House dust mite; KO, knockout; HC, Healthy controls; qRT-PCR, Quantitative real-time polymerase chain reaction; ROR, Retinoic acid-related orphan receptor; SDS, Sodium dodecyl sulfate; shRNA, Short-hairpin ribonucleic acid; TRC, The RNAi Consortium; TRM, Tissue-resident memory T; Th1, Type 1 helper T cells; Th2, Type 2 helper T cells; Th17, Type 17 helper T cells; TSLP, Thymic stromal lymphopoietin

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Research in context

Evidence before this study

Past studies have shown that patients with atopic dermatitis have the tendency of developing atopic march, the progression of atopic dermatitis in infancy and early childhood to subsequent airway hyperresponsiveness such as allergic rhinitis and asthma, in the later stages of their lives. Th 17 inflammation, which is often observed in systemic inflammation, has been proposed to be involved in atopic march. As yet, the mechanism to explain how this reaction induces atopic march has not been identified and a biomarker to predict atopic march is still unknown.

Sources

We performed a literature search in the PubMed database using the terms "atopic dermatitis" and "atopic march" or "allergic march" or "systemic inflammation" and "pathogenesis", or "biomarker", or "*Dermatophagoides*", or "*Dermatophagoides farinae*", or "der f", or "der f fraction", or "T cell", or "IL-17", or "Th17", or "immune" and/or "response", or "psoriasis" and/or "lipid metabolism", and/or "fatty acid metabolism", and/or "fatty acid binding protein 5", or "epidermal fatty acid binding protein", or "fatty acid binding protein 4" to find evidences before and while we are undertaking this study.

Added value of this study

A heatmap using a whole genome transcriptome displayed increased expression of genes related with fatty acid metabolism in the atopic march group when compared with the atopic dermatitis group without atopic march and with healthy controls. Among them, fatty acid binding proteins families, especially fatty acid binding protein 5-related genes, were most highly expressed. Consistently, increased fatty acid binding protein 5 expression was confirmed in human skin samples and T cells with atopic march and murine atopic march model's skin, lung and T cells, in accordance with increased IL-17A level, when compared with atopic dermatitis samples and healthy controls. Knockdown of fatty acid binding protein 5 in T cells inhibited IL-17A expression. Direct correlation was observed between fatty acid binding protein 5 expression and IL-17A level. Overall, the results indicate that 'fatty acid binding protein 5' may serve as a prudent biomarker to explain the progression of atopic march in atopic dermatitis patients, acting by directly promoting Th17 inflammation.

Implications of all the available evidence

Our findings imply the possibility to predict the atopic march progression with an identifiable biomarker 'fatty acid binding protein 5', in relation to the past findings regarding the mechanism of inflammation transfer within the body system by Th17 inflammation. This will help clinicians to explain the in-depth mechanism of atopic march, predict atopic dermatitis patients with higher risk of atopic march progression and provide more detailed information to such patients. Furthermore, this study will be useful for researchers in various departments and academia regarding the involvement of fatty acid binding protein 5 in Th17-mediated systemic inflammatory diseases.

1. Introduction

Atopic dermatitis (AD) is a chronic, relapsing inflammatory skin disease that limits the patient quality of life [1,2]. AD is also considered a systemic disease [3] that is associated with autoimmune disorders [4] and involvement of the ocular [5], gastrointestinal [6,7], and renal systems [8]. The strongest evidence for systemic involvement is the phenomenon called "atopic march" (AM) [9].

The concept that AM represents AD as a systemic disease is supported by cross-sectional and longitudinal studies [10–13]. Importantly, atopy is the familial or personal propensity to produce systemic IgE antibodies with sensitization to inhaled and food allergens [14]. Atopy links the conditions of AD, allergic rhinitis (AR), and allergic asthma (AA) [15-17]. Classically, AD constitutes only a part of the atopy complex, which encompasses skin changes usually combined with airway involvement, including the lung and nasal mucosa, leading to the progression from atopic eczema to respiratory allergic diseases [15,18]. Although these conditions can develop independently, early eczematous skin lesions and later-onset respiratory disorders share a common pathogenesis [18].

While the exact pathomechanism of AM has not been identified, several possibilities have been previously proposed. One potential mechanism of AM pathogenesis posits that a defective skin barrier during childhood eczema allows entry of high-molecular-weight allergens, bacteria, and viruses, eventually causing airway hyperresponsiveness (AHR) [19,20]. Importantly, AHR requires the involvement of T cells [21]. Furthermore, epicutaneous entry of environmental allergens through barrier-disrupted skin induces thymic stromal lymphopoietin (TSLP) expression and is strongly associated with the induction of type 2 helper T cells (Th2). Th2 cells further contribute to airway sensitization and inflammation [22,23].

Although most previous AM studies have focused on Th2 responses, a study showed that interleukin (IL)−17A signaling might also contribute to AM pathogenesis [24]. Specifically, allergen-induced IL-6 trans-signaling activated $\gamma\delta$ T cells and promoted both Th2- and Th17-mediated airway inflammation [25]. IL-6 and other cytokines also stimulated Th17 differentiation. Taken altogether, these data suggest that Th17 responses may contribute to AM development even though the underlying mechanism of AM in relationship with Th17 remains unclear.

The aim of this study is to elucidate the Th17-related mechanism of AM pathogenesis and to identify a biomarker to predict the high-risk patients for AM progression among AD patients. Therefore, in this study, we developed AM mice models and sampled skin and serum from healthy controls (HC), AD and AM patients to elucidate the Th17-related mechanism. Whole genome transcriptome analysis was done to find a target protein which promotes Th17 responses and gene expressions were analyzed using microarray and quantitative PCR. Immunofluorescence was performed to visualize the target protein in tissue samples. A knockdown experiment was done to clarify the direct relationship between our candidate protein and Th17 response. Our data confirmed the Th17-related mechanism of AM in a relation with a protein named fatty acid binding protein 5 (FABP5), which may be a novel biomarker to predict AM progression in AD patients.

2. Materials and methods

2.1. Patient selection

Patients diagnosed with AD according to the Hanifin and Rajka criteria [26] were enrolled in Department of Dermatology, Severance Hospital, Yonsei University Health System. AD can be categorized into extrinsic AD, caused by entry of an external allergen, and intrinsic AD that occurs regardless of allergy. This can be diagnosed with

Table 1
Demographics of the subjects enrolled in the study.

	AD	AM	HC
Total (n)	50	50	21
Male	23	26	11
Female	27	24	10
Age (Mean)	25.98 ± 6.68	27.42 ± 6.12	22.33 ± 2.75
Age (Median, Range)	26 (20–49)	27 (20–45)	22 (20–29)
Family history	38.89%	43.33%	–
EASI score	24.52 ± 9.02	26.32 ± 8.77	–
Total IgE	2231.73 ± 1681.91	2430.75 ± 1701.33	–
Eosinophils (%)	6.48 ± 3.91	6.81 ± 4.16	–
Der f (+)	82.0%	92.0%	–

Abbreviations: AD, atopic dermatitis; AM, atopic march; HC, healthy control; EASI, eczema area and severity index; Der f, *Dermatophagoides farinae*. Results are expressed as mean±standard deviation.

total IgE test (IgE≥150UI/ml) and only extrinsic AD patients were included [27], since intrinsic AD is not related to AM. Enrolled patients were divided into two groups: those with AD only (AD group) and those who also had AR and/or AA (AM group). HC were also recruited. The diagnosis of AR and AA were confirmed by allergists (authors Kyung Hee Park and Jung-Won Park) according to the ARIA (Allergic Rhinitis and its Impact on Asthma) and GINA (Global Initiative for Asthma) guidelines, respectively. For the sample size, as this is an exploratory study in which the sample size calculation is difficult, previous similar studies [28,29] were reviewed and a similar sample size was determined. Demographics of enrolled subjects are described in Table 1 and a study flow diagram is shown in Fig. 1. Informed consent was obtained from all subjects, and the protocols were approved by the Institutional Review Board of Severance Hospital (no. 4–2013–0624).

2.2. Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Department of Laboratory Animal Resources, Yonsei Biomedical Research Institute, Yonsei University College of Medicine (IACUC No. 2016–0007). Female NC/Nga mice (6 weeks old) were purchased from SLC Japan (Shizuoka, Japan). Mice were kept under controlled humidity (40%) and temperature (22 ± 2 °C) conditions. HDM ointment was purchased from Biostir Inc. (Kobe, Japan). One gram of ointment contains 234 μg of Der f 1 (a group 1 major allergen of *Dermatophagoides farinae*), 7 μg of Der f 2, and 134.4 mg of other proteins.

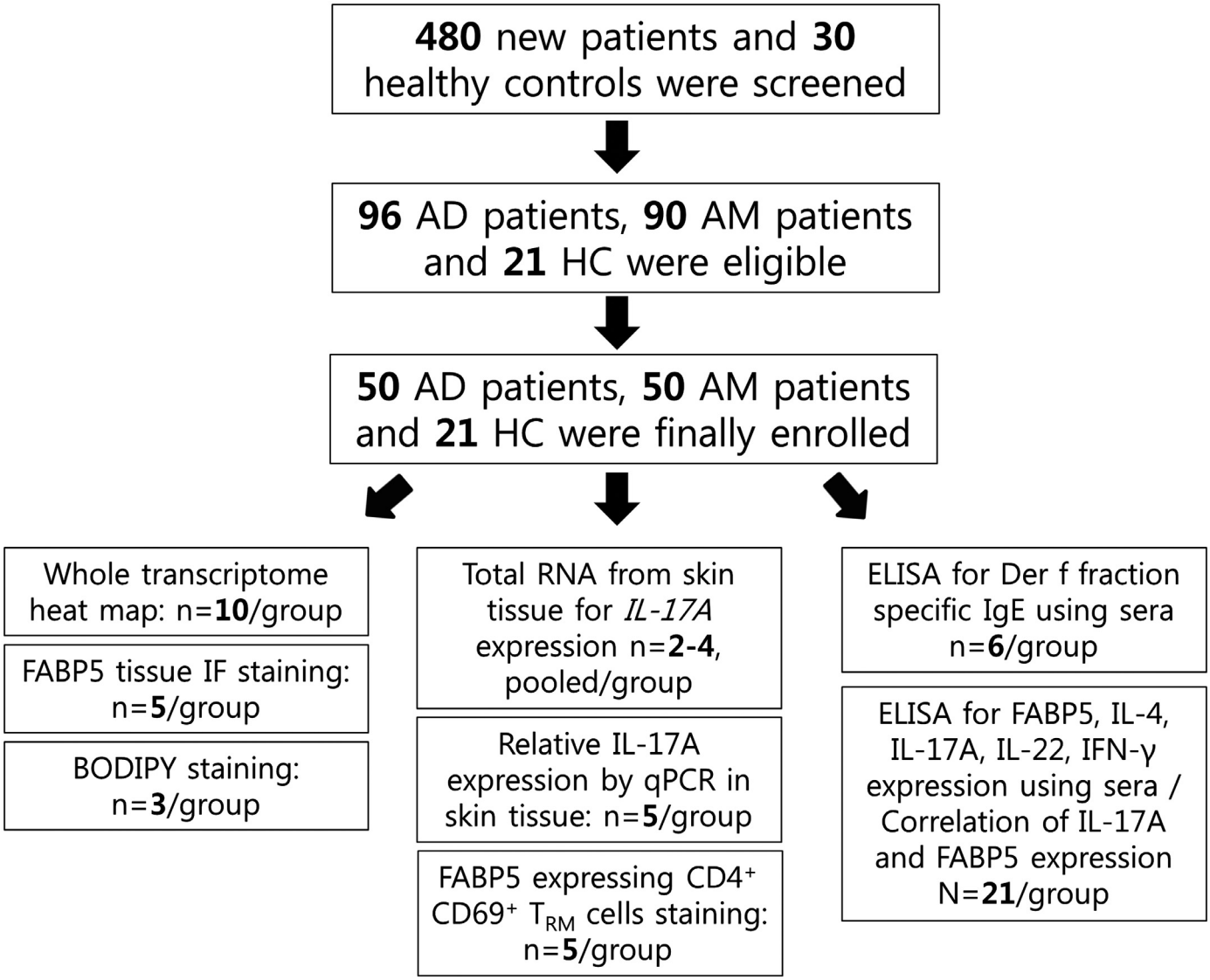

Fig. 1. Study flow diagram of atopic dermatitis (AD), atopic march (AM) and healthy control (HC) in the study.

2.3. AM model development in NC/Nga mice

A total of 33 NC/Nga mice were used: 5 as HC, 12 to induce skin lesions only (AD), and 16 to develop AM. In the first week, all NC/Nga mice were anesthetized with 3−5% isoflurane using a vaporizer (VetEquip Inc., Livermore, CA) and then their dorsum was shaved using a razor. Hair was completely removed using hair removal cream once per week. To disrupt the skin barrier and induce skin inflammation, we applied 200 μL of 4% sodium dodecyl sulfate (SDS) on the back and posterior auricular areas. After 2 h, 100 μg of HDM ointment was applied. Epicutaneous challenges with SDS and HDM were performed twice per week for 6 weeks (12 applications total). To induce AM, 100 μg of *D. farinae* antigen was delivered intranasally daily for the last 3 d (Supplementary Fig. S1). Twenty-four hours after the last challenge, the skin, blood, lymph nodes, spleens, and lungs were collected to evaluate immune responses.

2.4. Microarray analysis

Total RNA in human skin biopsy samples was isolated from HC (n = 10), AD (n = 10) and AM (n = 10) patients. Total RNA derived from T cells was obtained from 16 patients with AD and 16 patients with AM. All microarray analyses were performed at Macrogen (Seoul, Korea). RNA integrity and purity were evaluated using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE) and Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA was amplified and purified using a TargetAmp-Nano Labeling Kit for Illumina Expression BeadChip (Epicentre, Madison, WI, USA) for biotinylated cRNA according to the manufacturer's instructions. 400 ng of total RNA was reverse-transcribed using T7-oligo (dT) promoter primers. Following second-strand cDNA synthesis and purification, cRNA was quantified using an ND-1000 spectrophotometer (NanoDrop). Labeled cRNA was hybridized to each Human HT-12 v4.0 Expression Beadchip for 17 h at 58 °C according to the manufacturer's instructions (Illumina Inc., San Diego, CA). Array signal detection was performed using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) following the bead array manual. Arrays were scanned with an Illumina bead array reader confocal scanner. Raw data were extracted using the manufacturer-provided software (Illumina GenomeStudio v2011.1; Gene Expression Module v1.9.0). All data analysis and visualization of differentially expressed genes was performed using R 3.1.2 (www.r-project.org). Moreover, two GEO data sets (GSE124700 (2 HC samples, 1 AD sample) GSE124701 (1 AD sample) were utilized for pattern analyzing of gene expression among patients' groups. Prior to data assay, we redefined diagnoses of atopic disease patients according to history of allergic disease in their medical reports. Microarray data were uploaded to the Gene Expression Omnibus (accession code GSE146352 for human skin and GSE146356 for human T cell).

2.5. Functional study using short-hairpin(sh) RNA interference

2.5.1. Lentiviral plasmid vectors

MISSION pLKO.1-puro lentiviral plasmid containing mouse Fabp5, human FABP4, FABP5 shRNA and nontargeting shRNA control were obtained from Sigma-Aldrich. The RNAi Consortium numbers for shRNA used in this study are TRCN0000011895 (Fabp5 for mouse), TRCN0000059702 (FABP5 for human), TRCN0000059621 (FABP4 for human) and SHC002 (nontargeting shRNA control). shRNA inserts were verified using Sanger sequencing. For packing and envelopment, pΔVPR and pVSVG plasmids were provided by Dr. Jun-Young Seo (Yonsei University College of Medicine).

2.5.2. Lentiviral packing, production, and transduction

Murine shRNA Fabp5 or human shRNA FABP5 sequence containing and not containing recombinant lentiviruses were produced by transient transfection of HEK293T cells plated in 100-mm dishes. pLKO.1-Puro-shLenti (8 μg), pΔVPR (8 μg), and pVSVG (8 μg) plasmids were co-transfected into HEK293T cells using Lipofectamine 3000 (Thermo Fisher Scientific), according to manufacturer's instructions. The culture supernatants containing virus particles were harvested every 24 h post-transfection and filtered through 0.45-μm-pore filters. HaCaT keratinocytes, human PBMCs, and murine splenocytes were transduced with lentivirus through spinfection, as previously reported [30].

2.6. Statistical analysis

A minimum of two independent experiments were conducted to obtain data. Statistical analysis was conducted using SPSS version 20 (SPSS Inc., Chicago, IL, USA). Statistical differences were considered significant at $P<0.05$. The correlation of nonparametric paired data was tested using Spearman's rho. The significance between two groups was evaluated using unpaired t-tests and among multiple groups was evaluated using one-way analysis of variance (ANOVA) statistics with Tukey's multiple comparison test or two-way ANOVA with Dunnett's multiple comparison test. Graphs were expressed as mean±standard error of the mean using GraphPad Prism, version 6.01 (GraphPad Software, San Diego, CA, USA).

Additional methods are available in the Supplementary Methods file.

3. Results

3.1. AM is strongly associated with fatty acid metabolism through FABP5 induction

First, we analyzed the clinical phenotypes of AD patients with and without AM (Table 1). We compared the severity based on the eczema area and severity index (EASI), serum total IgE levels, blood eosinophil levels, and the presence of family history. Importantly, there was no statistically significantly difference between the two groups based on clinical characteristics. Results were similar to our previous study [31,32].

Next, we performed whole-transcriptome analysis of HC, AD, and AM human skin samples, which revealed differential gene expression between the three groups (Fig. 2A). Interestingly, the genes that were more elevated in AM patients than AD were related to fatty acid metabolism (Fig. 2B and Supplementary Figs. S2 and S3). This suggested a possible link between fatty acid metabolism and atopic march in AD. Thus, we focused further on genes related to fatty acid-binding proteins (FABPs) in our data sets. FABP5 and FABP5-like (FABP5L) genes were the most highly expressed genes in the FABP family from AM skin samples relative to AD (Fig. 2C). Consistently, mRNA expression of *FABP5*, determined-by RT-PCR was statistically significant in the AM group versus AD, but it was also significantly different between the AD and HC groups (P = 0.0261 and P = 0.0323, respectively, one-way ANOVA with Tukey's multiple comparison test, Fig. 2D).

Immunofluorescence staining further confirmed that AM patients expressed clearly enhanced FABP5, also known as epidermal-FABP (E-FABP), in the epidermis of AM skin compared to AD or HC skin (Fig. 2E and F). Furthermore, BODIPY staining for intracellular lipids revealed that AM skin had distinctly more lipid droplets than AD or HC skin (Fig. 2G and H). These droplets were distributed not only in the epidermis but also in the dermis (Supplementary Fig. S4), suggesting an association between lipid metabolism and significant inflammation in AM compared to AD skin.

Because survival of T cells is known to require lipid metabolism through FABP4/5 expression [33], we compared FABP family gene expression between AD and AM patients by using isolated T cells (Supplementary Fig. S5). Consistent with our previous results using skin samples, T cells from AM patients strongly expressed FABP5/

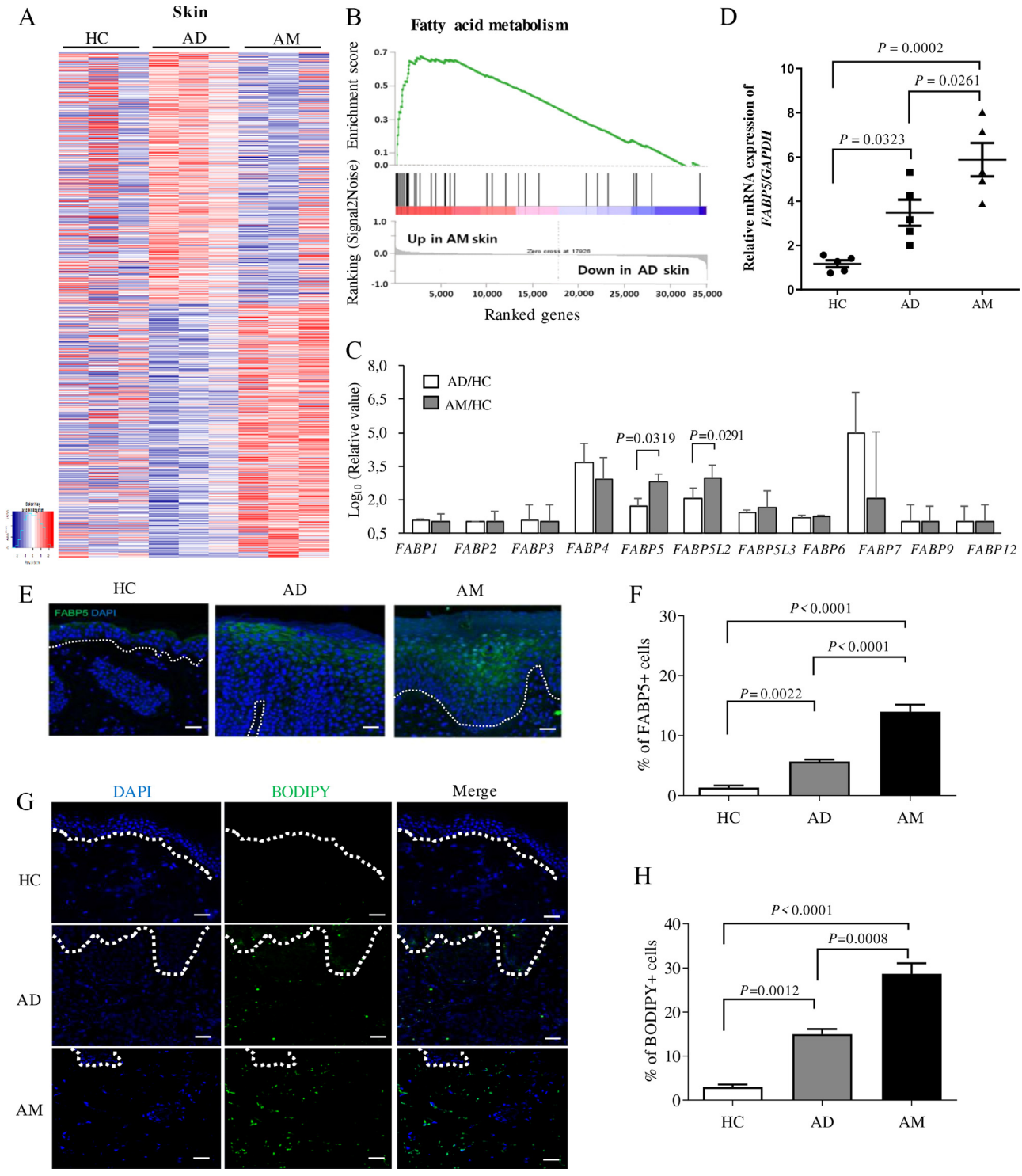

Fig. 2. Atopic march (AM) is associated with increased expression of fatty acid-binding protein 5 (FABP5). **(A)** Whole-transcriptome heat map of Healthy Control (HC), atopic dermatitis (AD), and AM skin (n = 10 per group). Total RNA derived from skin biopsies was pooled 2–4 samples (depending on RNA amount). **(B)** Fatty acid metabolism-related genes upregulated in AM skin. A list of genes are provided in Supplementary Table S1. **(C)** Bar plots displaying FABP family gene expression changes in AD and AM skin. Relative values were normalized to HC skin. **(D)** Relative *FABP5* expression determined by quantitative PCR (qPCR), and **(E)** Representative immunofluorescent FABP5 staining and **(F)** the intensity of FABP5 expressions in HC, AD, and AM skin tissues (n = 5/group). **(G)** BODIPY staining and **(H)** intensity of HC, AD and AM skin (n = 3/group). Dotted line represents the epidermal-dermal boundary. Scale bar represents 50 μm. All experiments related to **(D), (E)** and **(G)** were independently performed three times.

FABP5L2 genes. However, the expression of FABP4, or adipocyte-FABP, was only slightly increased in T cells (Supplementary Fig. S5) and in the serum of AM patients (Supplementary Fig. S6A), and it was not increased in AM skin (Supplementary Fig. S6B). Thus, it showed that FABP5 induction in AM skin may be related to extended inflammation in fat-rich environments, like the skin, through long-term survival of T-cells [33].

3.2. *FABP5 directly upregulates Th17 in AM*

To explore the type of inflammation associated with AM skin, we further analyzed Th1/ Th2/ Th17/ Th22 inflammation-related genes [34] in HC, AD, and AM human skin samples. This revealed that AM was associated predominantly with increased expression of Th17-associated genes (Fig. 3A), but Th1/ Th2/ Th22-associated

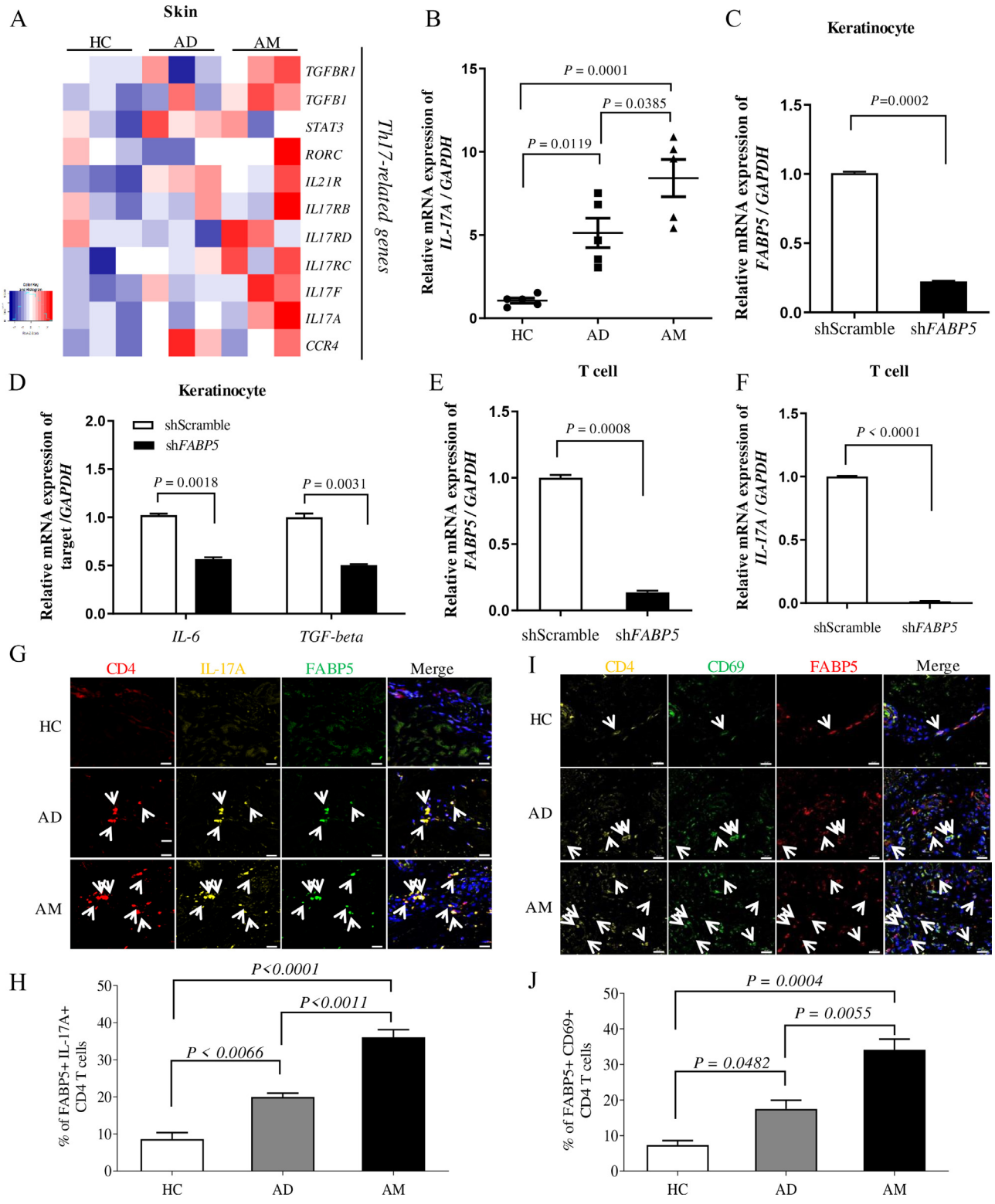

Fig. 3. FABP5 enhances type 17 helper T cell (Th17) expression in AM. **(A)** Heat map of differentially expressed Th17 cell-related genes between human HC, AD, and AM skin (n = 10/group). Total RNA derived from skin biopsies was pooled 2–4 samples (depending on RNA amount). **(B)** Relative IL-17A expression determined by qPCR in HC, AD, and AM skin tissues (n = 5/group). **(C)** Confirmation of FABP5 knockdown in shFABP5-transduced human HaCaT keratinocytes. **(D)** Real-time PCR results of Th17-inducing cytokines (IL-6, transforming growth factor [TGF]-beta) depending on FABP5 expression in HaCaT cells. **(E)** Confirmation of FABP5 knockdown in shFABP5-transfected T cells from AM patients. **(F)** Real-time PCR results showing the effect of FABP5 knockdown on IL-17A expression in AM T cells. **(G)** Immunofluorescence of FABP5 and **(H)** percentage of FABP5$^+$ cells in CD4- and IL-17-producing T cells and **(I and J)** FABP5 expressing CD4$^+$ CD69$^+$ tissue-resident memory T (T_{RM}) cells in human HC, AD, and AM skin tissues (n = 5/group). Scale bar represents 20 μm. Arrowheads indicate co-stained cells. All data, except the heatmap, are representative of three independent experiments.

Table 2
Clinical characteristics of HC, AD and AM mice.

	HC (n = 5) Mean (point estimates)	95% CI	AD (n = 12) Mean (point estimates)	95% CI	AM (n = 16) Mean (point estimates)	95% CI	p-value (AD vs AM)
Erythema	0	—	1.083	0.5113 - 1.655	1.625	1.1995 - 2.055	0.2269
Scarring	0	—	1.667	1.354 - 1.980	2.215	1.742 - 2.508	0.1208
Edema	0	—	1.500	1.168 - 1.832	1.563	1.289 - 1.836	0.9383
Excoriation	0	—	1.833	1.377 - 2.289	1.938	1.482 - 2.393	0.9288
SCORAD	0	—	6.200	5.388−7.012	7.125	6.326−7.924	0.1762

Subjective symptoms including pruritus and sleep loss were excluded from the calculation.

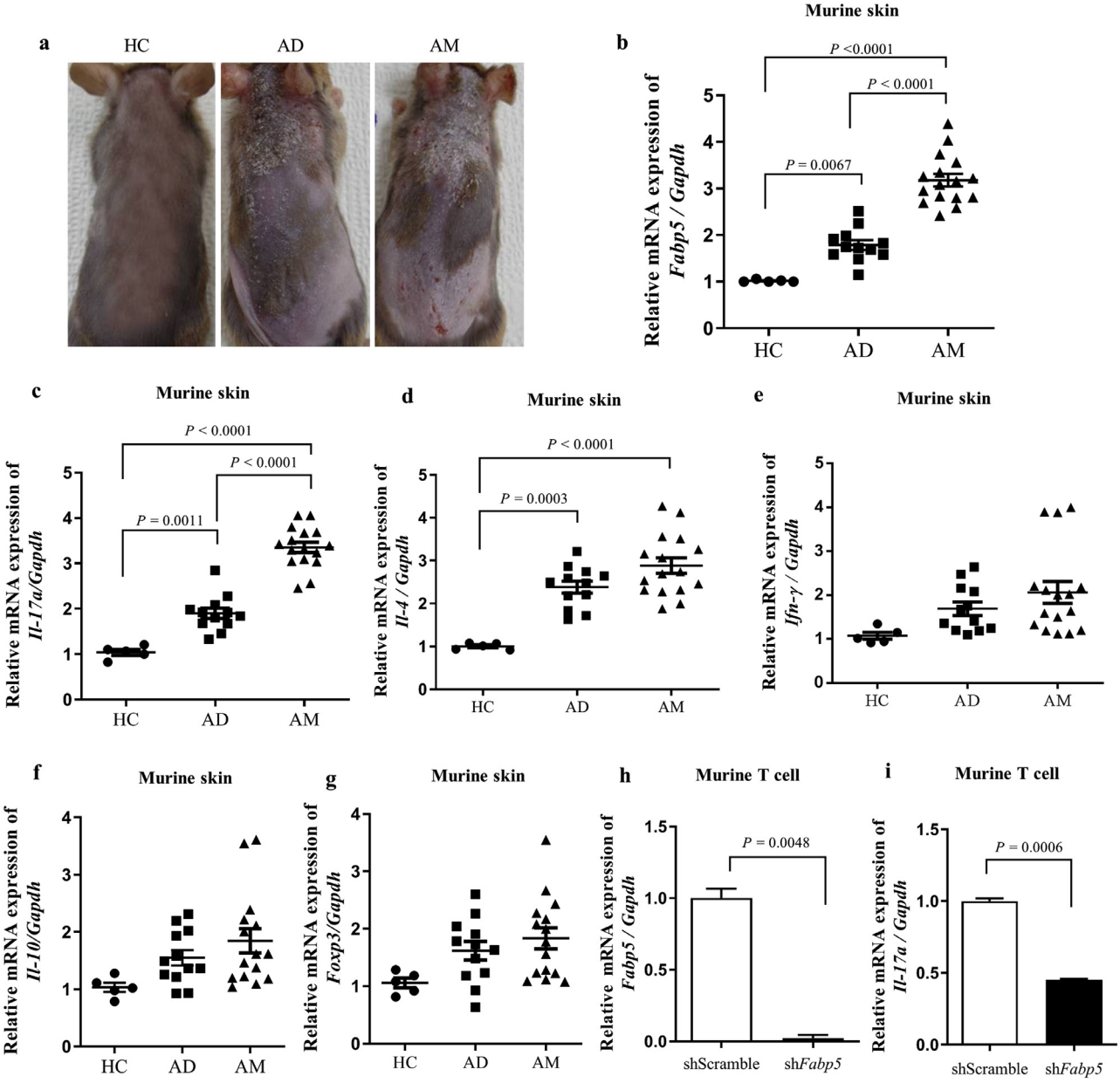

Fig. 4. Overexpression of *Fabp5* and Th17-related genes in AM mice. **(A)** Representative photographs of HC, AD, and AM mice after 6 weeks of challenge. qPCR results of **(B)** *Fabp5*, **(C)** *Il17a*, **(D)** *Il4*, **(E)** *Ifn-gamma*, **(F)** *Il10*, and **(G)** *Foxp3* expression in dorsal skin from HC, AD and AM mice (n = 5/group). **(H)** Confirmation of *Fabp5* knockdown in normal murine T cells. **(I)** *Il17a* expression in FABP5-shRNA-transduced normal murine T cells. ns, not significant; Bar graphs are representative of three independent experiments.

genes were not increased (Supplementary Fig. S7). Specifically, AM skin had statistically significantly higher relative expression of IL-17A compared to HC and AD (P = 0.0001 and P = 0.0385, respectively, one-way ANOVA with Tukey's multiple comparison test, Fig. 3B).

As AM patients expressed high levels of FABP5 in both epidermis (keratinocytes) and dermis (T cells) (Fig. 2E, F and Supplementary Fig. S5), we performed functional studies using shRNA interference of FABP5 in keratinocytes (HaCaT cells) and T cells. FABP5 knockdown in HaCaT cells (Fig. 3C) resulted in significant reduction of the expression of Th17-inducing cytokines, including IL-6 and transforming growth factor (TGF)-β (P = 0.0018 and P = 0.0031, respectively, unpaired t-test, Fig. 3D). However, this knockdown did not significantly affect TSLP expression that enhances Th2 response (Supplementary Fig. S8), FABP5 knockdown in human AM T cells also significantly inhibited IL-17A expression ($P < 0.0001$, unpaired t-test, Fig. 3E and F).

Furthermore, FABP5-expressing $CD4^+$ T cells concurrently produced IL-17A and were found more abundantly in human AM skin when compared with those from HC and AD skin (Fig. 3G and H). These $FABP5^+$ Th17 cells expressed CD69, indicating that they were tissue-resident memory T (T_{RM}) cells (Fig. 3I). T_{RM} cells were significantly more abundant in AM skin than HC and AD (Fig. 3J). Th17 T_{RM} cells were enriched in the skin of AM patients, possibly because FABP5 directly induces Th17 polarization.

3.3. Increased FABP5 and IL-17A expression in an AM murine model

To further evaluate the functional role of FABP5 in AM, we developed an AM murine model using an established AD mouse system [35]. Specifically, NC/Nga mice received house dust mite (HDM) ointment on the back and ear for six weeks and then the AM group mice received intranasal HDM extracts for 3 days. The experimental timelines for each murine model are shown in supplementary Fig. S1 Representative photographs of the mice are shown in Fig. 4A. Skin symptom severity was assessed using SCORAD (SCORing AD) and results are described in Table 2. Both the AD and AM murine models had significantly higher severity than HC, but severity of AD and AM skin did not statistically differ. AD and AM skin also had significantly higher relative *Fabp5* expression than HC (P = 0.0067 and $P < 0.0001$, respectively, one-way ANOVA with Tukey's multiple comparison test), and AM skin expressed more *Fabp5* than AD with statistical significance (Fig. 4B). AM skin also expressed significantly higher *Il-17a* than AD ($P < 0.0001$, one-way ANOVA with Tukey's multiple comparison test, Fig. 4C), but the two groups did not differ in terms of *Il-4, Ifn-γ, Il-10*, and Foxp3 expression (Fig. 4D–G), which are related with Th2 (*Il-4*), Th1 (*Ifn-γ*) and regulatory T cell (*Il-10, Foxp3*) responses. Finally, *Fabp5* knockdown in shRNA-transduced normal murine T cells (Fig. 4H) significantly inhibited *Il-17a*expression ($P < 0.0006$, unpaired t-test, Fig. 4I). Consistent with our human sample results, these results indicated that FABP5 drives Th17 polarization in AM.

3.4. FABP5 and IL-17A expression is strongly associated with lung inflammation in AM

To investigate the functional role of FABP5 in lung tissues from AM mice model, we assessed airway hyperresponsiveness (AHR) in AD and AM mice using the methacholine challenge test [36]. At 25 and 50 mg/mL methacholine, AM mice demonstrated significant AHR compared to AD ($P < 0.0001$, two-way ANOVA with Dunnett's multiple comparison test), clearly indicating an exaggerated AHR in AM mice (Fig. 5A). Lungs from AM mice also expressed distinctly more FABP5 than AD (Fig. 5B and 5C). Since FABP4 is known to be expressed in the lung of asthma patients [37], we visualized both FABP4 and FABP5 expression in lungs of AM mice. When visualized, FABP5 was primarily observed in airway epithelial cells and weakly in lung tissue, whereas FABP4 was localized to the lung tissue of AM mice (Supplementary Fig. S9).

Next, we compared the levels of Th17 and Th2 cytokines produced by $CD4^+$ T cells in the skin draining lymph nodes, spleens, and lungs. In AD and AM mice, IL-17A was significantly elevated in the lymph nodes relative to HC (Fig. 5D). This was also increased in AM mice relative to AD, although this difference was not statistically significant. In lung tissues, IL-17A expression was elevated in AM mice only (Fig. 5D) with statistical significance. IL-4, a Th2 cytokine, was also elevated in the lymph nodes of AD and AM mice, and especially high in AM mice with statistical significance; however, IL-4 was not increased in AM mouse lungs (Fig. 5E). This suggested that in AM mice, IL-17A is associated with strong inflammation involving the lungs and skin systemically. Finally, AM mice had significantly higher serum total IgE levels than HC and AD (Fig. 5F). This indicated that AM mice had higher HDM sensitization through both epicutaneous and intranasal routes relative to AD mice with epicutaneous sensitization only.

3.5. Dermatophagoides farinae *1 drives Th17 polarization in AM by inducing FABP5*

The only apparent clinical and laboratory difference between AD and AM patients was increased sensitization to *D. farinae* and coexistence of airway symptom (Table 1). Differences in sensitization according to mite allergen fractions have been reported previously in patients with respiratory allergies compared to those with AD and specifically, AD patients were mainly sensitized to Der f 1 [38]. Therefore, we measured Der f 1-specific IgE levels in the serum of HC, AD, and AM subjects (Fig. 6A). AD and AM patients had significantly higher Der f 1-specific IgE levels than HC ($P < 0.0001$, one-way ANOVA with Tukey's multiple comparison test), and the difference between AD and AM patients was also significant ($P < 0.0001$, one-way ANOVA with Tukey's multiple comparison test). As AD patients are sensitized to other mite allergen fractions [38,39], we also evaluated IgE levels specific to Der f 10, 11, and 14; however, these did not significantly differ between groups (Fig. 6B–D).

Next, we co-cultured dendritic cells (DCs) with $CD4^+$ T cells and then treated the cultures with various Der f fractions, including whole Der f extracts (Der f), Der f 1, 2, 6, 11, and 14, to find direct evidence of a relationship between allergen fraction and inflammatory reaction in three groups. In cells from AM patients, Der f 1 treatment significantly elevated IL-4 levels compared to cells from AD and HC subjects ($P < 0.0001$, two-way ANOVA with Dunnett's multiple comparison test; Fig. 6E), further supporting that AM patients are sensitized to Der f 1. Der f 14 treatment also significantly increased IL-4 in the AD and AM groups relative to HC ($P < 0.0001$, two-way ANOVA with Dunnett's multiple comparison test); however, there was no difference between AD and AM (Fig. 6E).

Finally, we assessed relative *FABP5* expression in T cells from HC, AD, and AM subjects after treatment with whole Der f extracts (Der f), Der f 1, and Der f 14 (Fig. 6F). In these co-cultures, Der f 1 significantly increased *FABP5* expression in T cells from AM patients compared to those from HC and AD ($P < 0.0001$, two-way ANOVA with Dunnett's multiple comparison test); however, Der f 14 treatment did not significantly induce *FABP5* expression (Fig. 6F). Furthermore, in T cells from AM patients, Der f 1 treatment significantly induced IL-17A compared with those from AD ($P < 0.0001$, two-way ANOVA with Dunnett's multiple comparison test). Alternatively, Der f 14 produced lower levels of IL-17A in T cells from AM patients than Der f 1 treatment (Fig. 6G). Overall, these results suggested that in AM patients, Der f 1 drives Th17 polarization by inducing T cell FABP5 expression.

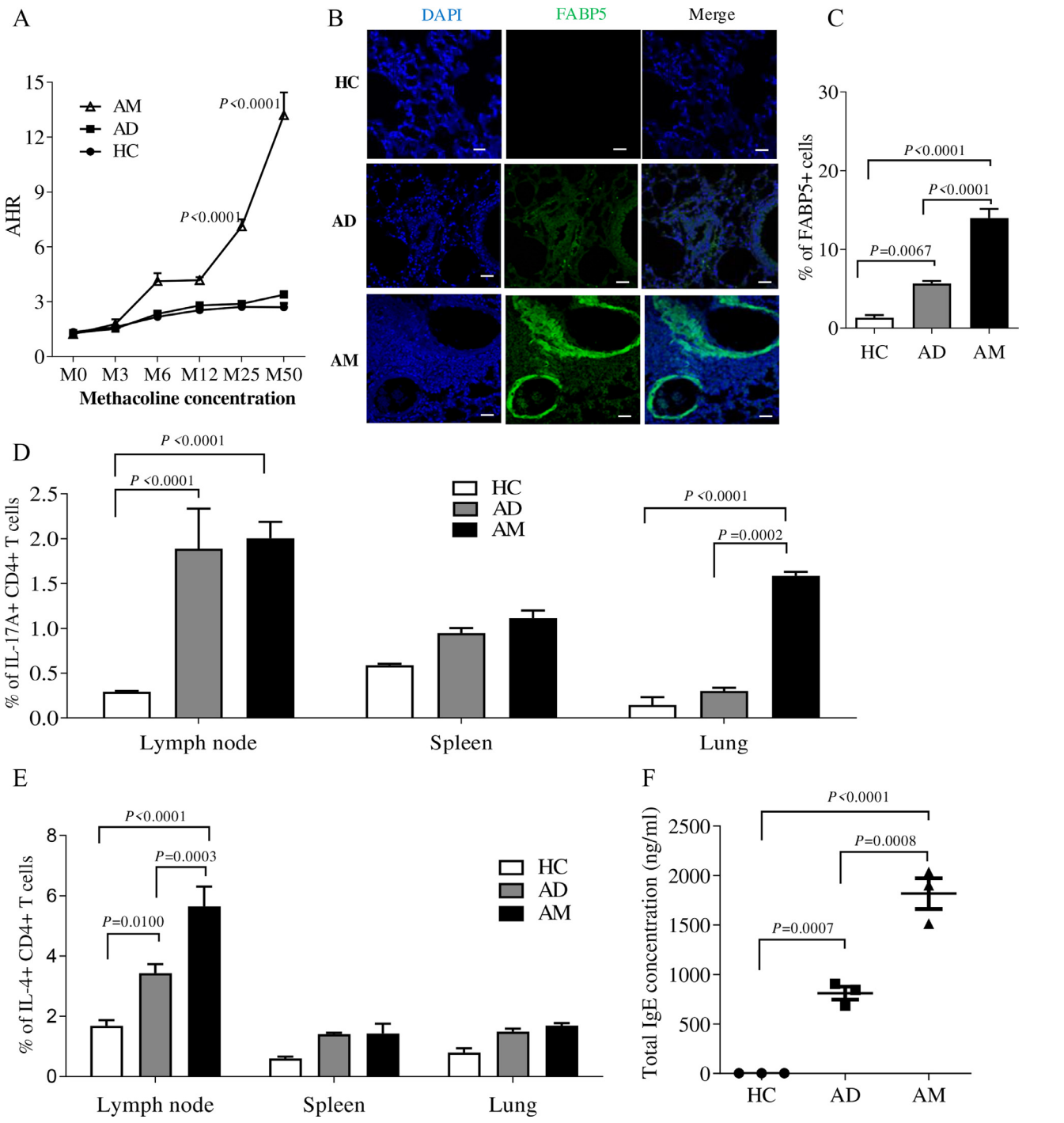

Fig. 5. Increased expression of FABP5 and IL-17A in lung inflammation of AM mice. **(A)** Evaluation of airway hyperresponsiveness (AHR) through a methacholine challenge in HC, AD and AM animal disease model (n = 3/group). **(B)** Representative confocal images showing increased FABP5 expression and **(C)** percentage of FABP5$^+$ cells in the lungs of HC, AD and AM mice (n = 3/group). Scale bar represents 50 μm. Summary data of intracellular staining in **(D)** CD4$^+$ IL-17a$^+$ and **(E)** CD4$^+$ IL-4$^+$ T cells from HC, AD, and AM mice (n = 3/group). **(F)** ELISA analysis of total IgE levels in HC, AD, and AM sera (n = 3/group). All experiments were independently performed three times with similar results.

3.6. FABP5 positively correlates with IL-17A

Finally, we compared serum FABP5 levels among those three groups using enzyme-linked immunosorbent assay (ELISA). Significant differences of serum FABP5 level were displayed not only between HC and AD sera, but also between AD and AM (Fig. 7A). We also used ELISA to evaluate group differences in the serum levels of IL-4, IL-17A, IL-22, and IFN-γ. Both AD and AM groups expressed significantly increased IL-4 relative to HC (P = 0.0001 and P = 0.0004, respectively, one-way ANOVA with Tukey's multiple comparison test, Fig. 7B), and there was no significant difference between AD and AM groups. Both AD and AM groups expressed significantly increased IL-17A versus HC group (P = 0.0430 and $P < 0.0001$, respectively, one-way ANOVA with Tukey's multiple comparison test) and the difference between AD and AM groups showed even greater statistical significance (Fig. 7C). Similarly, both the AD and AM groups expressed statistically significantly higher IL-22compared to HC, and no differences were observed between AD and AM (Fig. 7D). Even though both IL-17A and IL-22 are cytokines related to Th17 response, only IL-17A was significantly elevated in AM. This also suggested the direct relationship between FABP5 and IL-17A.

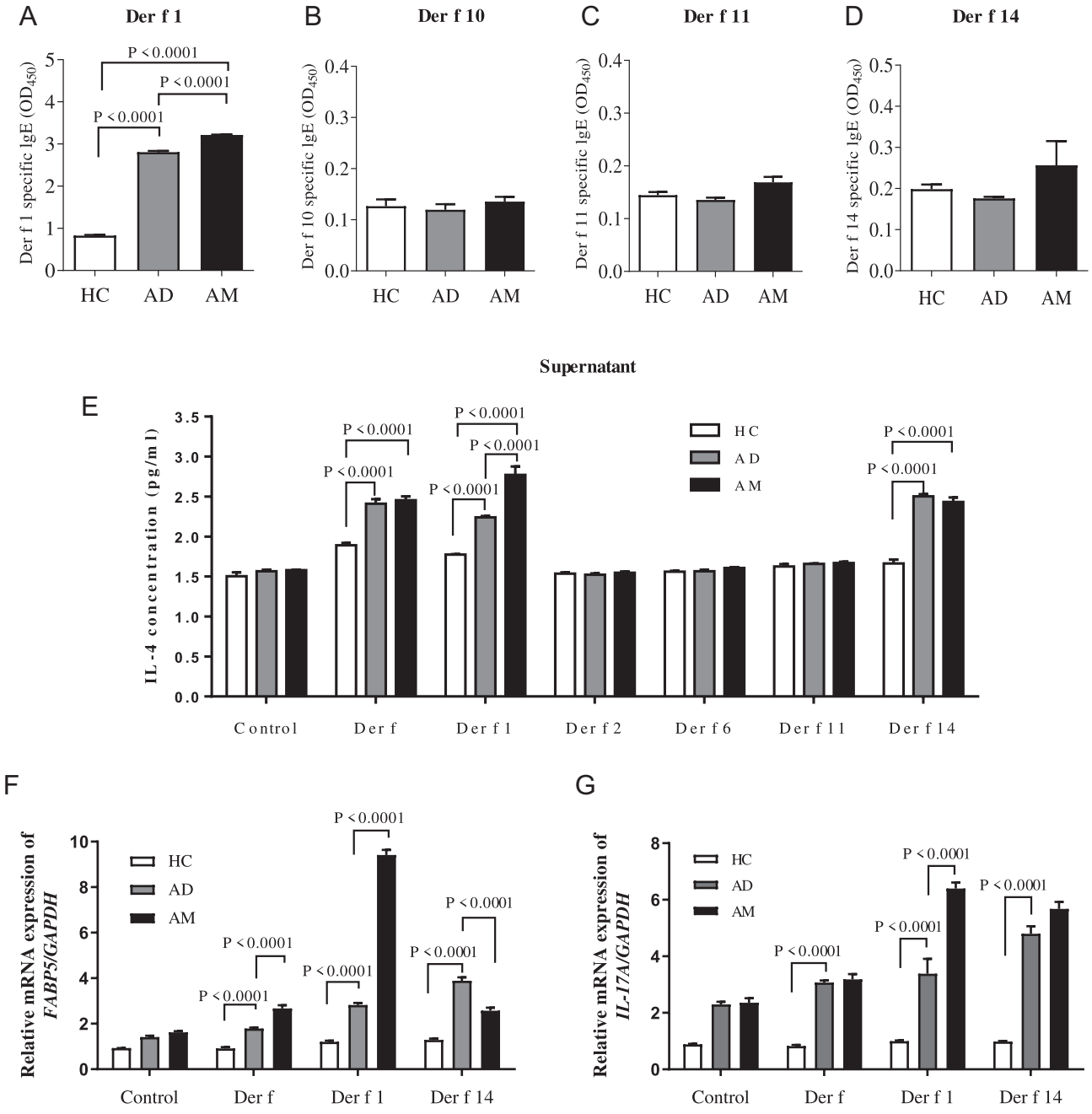

Fig. 6. Der f 1 mediates AM progression by increasing FABP5 expression. **(A-D)** ELISA for **(A)** Der f 1, **(B)** Der f 10, **(C)** Der f 11, **(D)** Der f 14-specific IgEs in human HC, AD, and AM sera (n = 6/group). **(E)** ELISA for IL-4 levels after co-culturing dendritic cells (DCs) and $CD4^+$ *T* cells. **(F, G)** qPCR results of the expression of **(F)** FABP5 and **(G)** IL-17A in T cells after treating DC/$CD4^+$ *T* cell co-cultures with Der f 1, Der f 14, and whole *Dermatophagoides farinae* extract (Der f) (n = 6/group). ns, not significant; All figures are representative of three independent experiments.

Thus, we evaluated the direct correlations of serum levels of FABP5 with each cytokine in HC, AD, and AM groups. FABP5 expression did not statistically correlate with IL-17A levels in HC and AD groups (Fig. 7F and G) but positively correlated with IL-17A in AM group (P = 0.041, Fig. 7H). However, FABP5 expression did not statistically correlate with IL-4, IL-22, and IFN-γ levels in HC, AD, and AM groups (Supplementary Fig. S10).

4. Discussion

While several studies support that AM is a systemic disease [3,9], the underlying pathomechanism remains unclear. Previous studies have demonstrated that AM is associated with increased IL-17A expression [40,41]. Systemic Th17 responses can be exacerbated by antigen challenge through epicutaneous sensitization even in the absence of Th2 responses, promoting airway inflammation and AHR [41]. Our RNA microarray analysis of human skin samples revealed that AM patients had a tendency toward increased expression of the IL-17 family, Th17-related cytokines. AM skin samples also demonstrated significantly increased expression of fatty acid metabolism-related genes. Moreover, AM patients expressed significantly pronounced *FABP5*, the only FABP family gene that appeared highly in AM skin samples compared to both HC and AD patients.

The skin provides a lipid-rich but nutrient-poor microenvironment, and lipids are required for permeable barrier function of cornified layers [42,43]. FABP5 modulates fatty acid uptake, transport, and

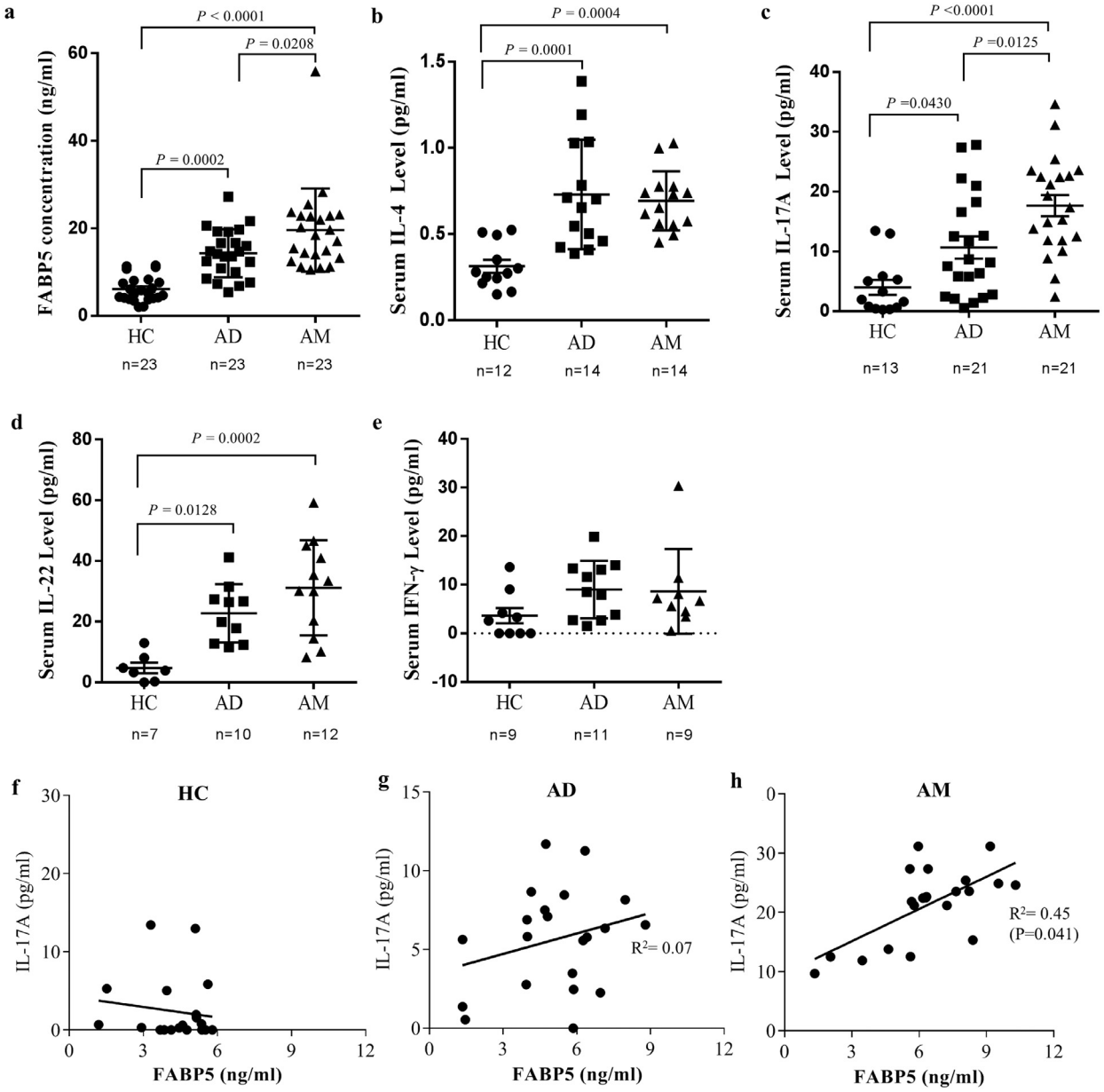

Fig. 7. Significant positive correlation between FABP5 and IL-17A in patients with AM. **(A)** FABP5 expression in HC, AD, and AM sera were measured using ELISA **(B-E)** The expressions of **(B)** IL-4, **(C)** IL-17A, **(D)** IL-22, and **(E)** IFN-γ were measured in the sera of HC, AD, and AM groups by ELISA. **(F-H)** Correlation of IL-17A expression and FABP5 levels in the sera of **(F)** HC, **(G)** AD, and **(H)** AM groups (n=21/group). ns, not significant; All experiments were independently performed three times with similar results.

metabolism [44]. FABP5 is known as E-FABP because it is detected in the granular layer of normal skin and establishes the skin barrier function. FABP5 is also called a psoriasis-associated FABP due to its enhanced expression in psoriatic skin, which is accompanied by hyperproliferation, impaired lipid metabolism, and abnormal differentiation [45]. A relationship is established between FABP5 and AD, as demonstrated in several studies; mass spectrometry of AD skin showed that both acute and chronic AD lesions express significantly more FABP5 [46]. This implies that FABP5 may play a role in AD [47]. Furthermore, extensive AM experiments comparing sputum and nasal lavage from four groups—AR, AR+AA, non-AR, and HC—revealed that the AR+AA group had significantly increased FABP5 expression. This may be interpreted as evidence that FABP5 contributes to airway remodeling and inflammation [48,49].

In our study, a heat map using whole-genome transcriptome analysis displayed increased fatty acid metabolism in AM. FABP5 and FABP5L genes were most highly expressed among the FABPs. Immunofluorescence analysis showed increased FABP5 expression in epidermis of AM and BODIPY study revealed increased lipid droplets in both epidermis and dermis, suggesting a role of FABP5 in the inflammatory response and an association of lipid metabolism in AM.

In adipose tissue, one of the main drivers of IL-17A responses is lipid spillover [50], suggesting that the Th17 pathway is linked to fatty acid metabolism. Interconnections between IL-17 and fat metabolism have also been studied [51]. Since IL-17A is particularly important in psoriasis to promote keratinocyte proliferation and inflammation [52], strong expression of FABP5 in psoriatic epidermis indicates a possible relationship between FABP5 and Th17 immunity [53]. The result that Asian AD phenotype includes Th17 polarization as well as Th2-predominant immune responses [54], also provides evidence of Th17 involvement in AD pathogenesis. We found that Th17-related genes and IL-17A were increased in AM skin samples. Furthermore, FABP5 knockdown inhibited IL-6 and TGF-β expression

from keratinocytes and consequently, IL-17A expression from T cells. Immunofluorescence analysis revealed that IL-17A-producing T cells expressed FABP5, emphasizing the association between FABP5 and IL-17A in AM skin. A previous study showed Th17 differentiation promoted by FABP5 expression in $CD4^+$ T cells [55]. It has been confirmed that IL-6 and TGF-β upregulate IL-17A production, and IL-6 sequentially induces IL-21 and retinoic acid-related orphan receptors (RORs) γt and α. This ROR activation subsequently induces T cell expression of IL-17A [56]. Taken altogether, these results emphasize the significance of FABP5 in inducing IL-17A in AM.

FABP5 expression was more intense in T_{RM} cells such as $CD4^+CD69^+$ T cells of AM patients. This implies that T_{RM} cells are not only important in tissue-specific inflammation [57,58], but also contribute to AM pathogenesis by interacting with FABP5. A recent study showed that $CD8^+$ T_{RM} cells expressed significantly higher Fabp4/5, and that Fabp4/5 deficiency reduced the long-term survival of T_{RM} cells [33]. We confirmed that human T_{RM} cells from AM skin had increased FABP5 expression when compared with AD and HC. Thus, these findings suggest an additional role of FABP5 in T-cell mediated inflammation in AM patients [59].

Although our AM murine model is artificial and may not perfectly resemble human AM, our study using Nc/Nga mice exhibited similar results with human skin and T cells. We developed AD and AM models separately and collected samples from skin, lungs, lymph nodes, and spleens. Just as was shown in human samples, the skin of AM mice expressed statistically significantly increased Fabp5 and *Il-17a*, a cytokine produced by Th17, compared to AD and HC. However, cytokine levels mainly produced by other types of inflammation such as Th2 (*Il-4*), Th1 (*Ifn-*γ) and regulatory T cell (*Il-10, Foxp3*) did not differ between AM and AD, implying a possible relationship between FABP5 and Th17 inflammation, rather than Th2, Th1 and regulatory T cell responses in AM progression.

Lung tissue from AM mice model also displayed strong FABP5 expression in airway epithelial cells and weak expression in lung tissue. FABP4 was observed in lung tissue only. Past studies have shown that FABP4 in lungs regulates the eosinophil recruitment and activation [60], and induces vascular remodeling and angiogenesis in allergic asthma [37]. Our study discovered that the first inflammatory reaction would begin when the allergen enters through the airway with disrupted barrier, where FABP5 is strongly expressed. The inflammatory reaction would then propagate to lung tissue where both FABP5 and FABP4 expression was confirmed. This may explain why serum FABP4 was also significantly increased in AM patients showing systemic inflammation compared with HC and AD group. As FABP4 and FABP5 are known to share genetic similarities, be present in macrophages and regulated in an essentially identical manner [44], we also tried to confirm whether there was any relationship between AM progression and FABP4, in addition to FABP5. However, in skin, FABP4 expression was increased in AD when compared with HC but no statistical significance was found between AD and AM. Thus, FABP4 is unlikely to be considered a possible marker to predict AM progression due the evidence of discrepancy in the serum and skin result.

We found that AM patients had higher sensitization to Der f 1 than AD patients. This corresponds with a recent study showing that Der f 1 was primarily sensitized in both respiratory and cutaneous atopic patients [38]. Such sensitization possibly occurred systemically through both epicutaneous and respiratory routes. Der f 1 also statistically significantly induced FABP5 and IL-17A expression in AM, whereas minor allergen fractions such as Der f 14 may be involved in AD pathogenesis with cutaneous sensitization only [38].

ELISA using human serum indicated a statistically significantly increased serum level of FABP5 in AM patients when compared with AD and HC group. IL-4, IL-22 and IFN-γ were elevated in both the AD and AM group without significant difference between the two groups, exhibiting similar results to mice samples. IL-17A expression

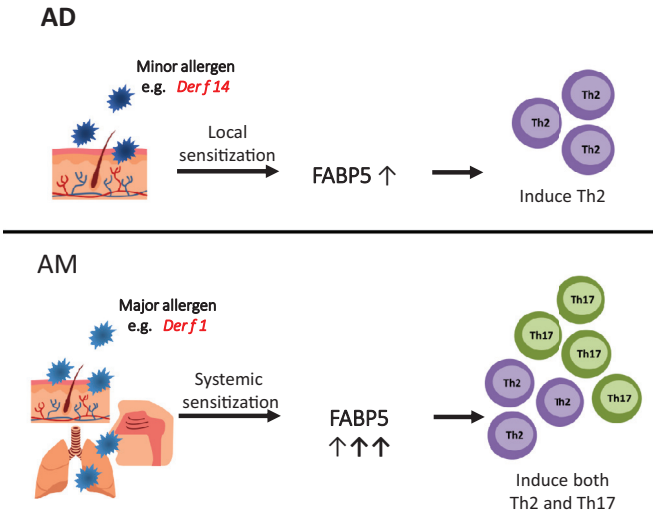

Fig. 8. Schematic diagram of the possible pathomechanism of atopic march **(AM)** and atopic dermatitis **(AD)**. This potential pathomechanism of AM development includes fatty acid-binding protein 5 (FABP5) induction and predominant environments of Th17-related cytokines following systemic sensitization through epicutaneous and respiratory routes to a major allergen from house dust mites, e.g., Der f 1.

was substantially higher in the AM group, even though it was elevated in both AD and AM groups. The result illustrates the constant relationship between AM and IL-17A. A noticeable correlation between the levels of FABP5 and IL-17A was only observed in AM group. FABP5 expression did not correlate much with IL-4, IL-22, and IFN-γ. IL-22 is a cytokine produced by Th17, thus these results imply FABP5 could be a potential biomarker for IL-17A expression within Th17 inflammation in AM.

The pathomechanism of AD can be explained by Th2-skewed immune responses that downregulate epidermal barrier protein expression and modulate IgE class switching, ultimately enhancing Th2 cell survival [61]. Th17 and IL-17A reportedly contribute to AD, especially in Asian [54] and pediatric [28,62] phenotypes. In this study, we observed that AM patients had a predominantly Th17 response through IL-17A production, which was further confirmed in our AM murine model. A schematic pathomechanism of AM through systemic sensitization, demonstrated in our study, is illustrated in Fig. 8.

Our findings imply the possibility to predict the risk of AM progression with an identifiable biomarker FABP5, in relation to the past findings regarding the mechanism of inflammation transfer within the body system by type Th17 inflammation, mediated by IL-17A. The importance of identifying a reliable biomarker has been strongly addressed at the recent National Institute of Allergy and Infectious Diseases workshop on “Atopic dermatitis and the atopic march: Mechanisms and interventions.”[63]. So far it has been agreed that the AM progression is highly relevant with risk factors such as AD severity, escalated transepidermal water loss, and polysensitization in infancy and childhood, but a prudent biomarker to indicate patients with higher risk of AM progression in earlier stage has not yet been identified. It is essential to predict such patients to explain the prognosis and to provide educational support and more effective treatment, eventually for the successful management [64]; that is why the Institute has delivered a striking message as to the finding of a biomarker at the workshop.

Limitations to our current study include the smaller sample size for experiments using skin tissue compared with serum studies, as it was difficult to recruit people who are willing to undergo skin biopsy for tissue collection. Although we have confirmed the direct relationship between FABP5 and IL-17 using an experimental knockdown approach, we did not conduct

overexpression experiments, which would provide extra confirmation. We decided here to take a step-by-step approach and prioritize our focus on confirming the link between FABP5 and Th17 response. This allows us to conduct future overexpression studies in more bias-free circumstances.

In conclusion, our data strengthens the link between AM and Th17 response by elucidating the correlation between the two when compared with HC and AD. Also, the role of FABP5 as a potential biomarker to predict high risk individuals for AM progression was highlighted, by confirming FABP5 overexpression induced Th17 inflammation in AM. These findings will provide a better understanding of the mechanism in AM progression and give a new insight into FABP5 as a potential biomarker in AM. It can also help clinicians and AD patients to acquire more information about prognosis of the disease and to establish an effective treatment plan.

Declaration of Competing Interest

The authors state no conflict of interest.

Author Contributions

This study was designed and coordinated by Kwang Hoon Lee and Chang Ook park, as the principal investigators who provided conceptual and technical guidance for all aspects of this research project and contributed writing the article. Jungsoo Lee contributed literature search, study design, human data collection, mice experiment, analysis, interpretation and drafting the article. Bomi Kim performed experiments including BODIPY and contributed figures, tables, writing methods and statistical analysis. Howard Chu contributed literature search, data collection, analysis, interpretation and writing the article. KeLun Zhang performed experiments, data collection and data analysis. Hyeran Kim performed ELISA using house dust mite (HDM) fractions and data analysis and Ji Hye Kim contributed mice experiment and data analysis. Seo Hyeong Kim contributed IF-staining and data analysis. Ji Yeon Noh performed literature search, whole gene transcriptome analysis and provided experimental guidance. ZhengWang Sun, Jongsun Lee, Kyung Yong Jeong, Kyung Hee Park, Jung-Won Park contributed methacholine test and AHR analysis using mice model and provided of HDM extracts. Youdong Pan and Thomas S. Kupper provided conceptual guidance for FABP experiments.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2020.102879.

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