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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1038/JID.2015.347.

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Elevated Galectin-10 Expression of IL-22-Producing T Cells in Patients with Atopic Dermatitis

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TO THE EDITOR

Atopic dermatitis (AD) is a highly pruritic, chronic, relapsing skin disease characterized by typically distributed skin lesions. Recently, subsets of T cells including T_H1, T_H2, T_H17, T_H22, and regulatory T (Treg) cells have been implicated as contributing factors to the immune dysregulation of AD. In this study, by using different quantitative proteomics, we identified differentially expressed proteins in $CD3^+$ T cells from patients with AD and healthy controls (HCs), and found that galectin-10 was elevated in IL-22-producing $CD4^+$ T cells in patients with AD.

This study was approved by the institutional review board, and written informed consent was obtained from each patient. A total of 45 patients with AD and 20 HCs were enrolled in this study. Diagnosis of AD was made according to the criteria of Hanifin and Rajka (1980). Patients were classified based on their allergic history, skin prick test, ImmunoCAP test, and serum IgE (Supplementary Table S1 online). Detailed methods were described in the Supplementary Methods online.

To study the differential protein expression from $CD3^+$ T cells in HCs and patients with AD, we performed LC/MS/MS analysis. Quantitative analysis based on the normalized spectral index (SI_N) of identified proteins (Griffin et al., 2010) resulted in Supplementary Table S2 online. Among the total 636 proteins identified, 75 and 81 showed higher expressions of five times more in $CD3^+$ T cells in patients with AD and in HCs, respectively. Extracted ion chromatograms of peptides from galectin-10 and S100A9 increased in patients with AD (Figure 1a) were presented with their MS/MS spectra (Supplementary Figure S1 online), confirmed by western blot analysis to be highly expressed in patients with AD (Figure 1b). Confocal laser microscopy also showed higher intensities of galectin-10 staining in $CD3^+$ T cells from patients with AD compared with HCs (Figure 1c).

As the upregulation of galectin-10 was mainly attributed to $CD4^+$ T cells (Figure 2a), we performed flow cytometry using $CD3^+CD4^+$ T cells in the following analysis. Galectin-10 was significantly upregulated in IL-22 expressing $CD3^+CD4^+$ T cells from patients with AD compared with HCs (Figure 2b). There was no significant difference of galectin-10 expression in $CD3^+CD4^+$ T cells producing other cytokines such as IL-17, IFN-γ, IL-4, or IL-10 (Figure 2b and Supplementary Figure S2 online). In the immunohistochemical staining, galectin-10 was highly expressed in $CD3^+$ T cells in AD skin, whereas its expression was nearly undetectable in the normal skin (Figure 2c). Interestingly, significant acanthosis was also observed adjacent to the dermal area where galectin-10^+ $CD3^+$ T cells were present, implying that epidermal hyperplasia in AD may result from galectin-10^+ T cells producing IL-22.

Next, we performed a correlation study between serum galectin-10 and various clinical and laboratory variables (Noh et al., 2014). The serum galectin-10 were significantly higher in patients with AD compared with HCs (Figure 2d, and Supplementary Table S3 online) showing a positive correlation between galectin-10 levels and eczema area and severity index score ($r = 0.409$, $P = 0.018$), and investigator's global assessment ($r = 0.553$, $P = 0.001$). No significant correlation was observed between serum galectin-10 and cell counts,

Abbreviations: AD, atopic dermatitis; HCs, healthy controls

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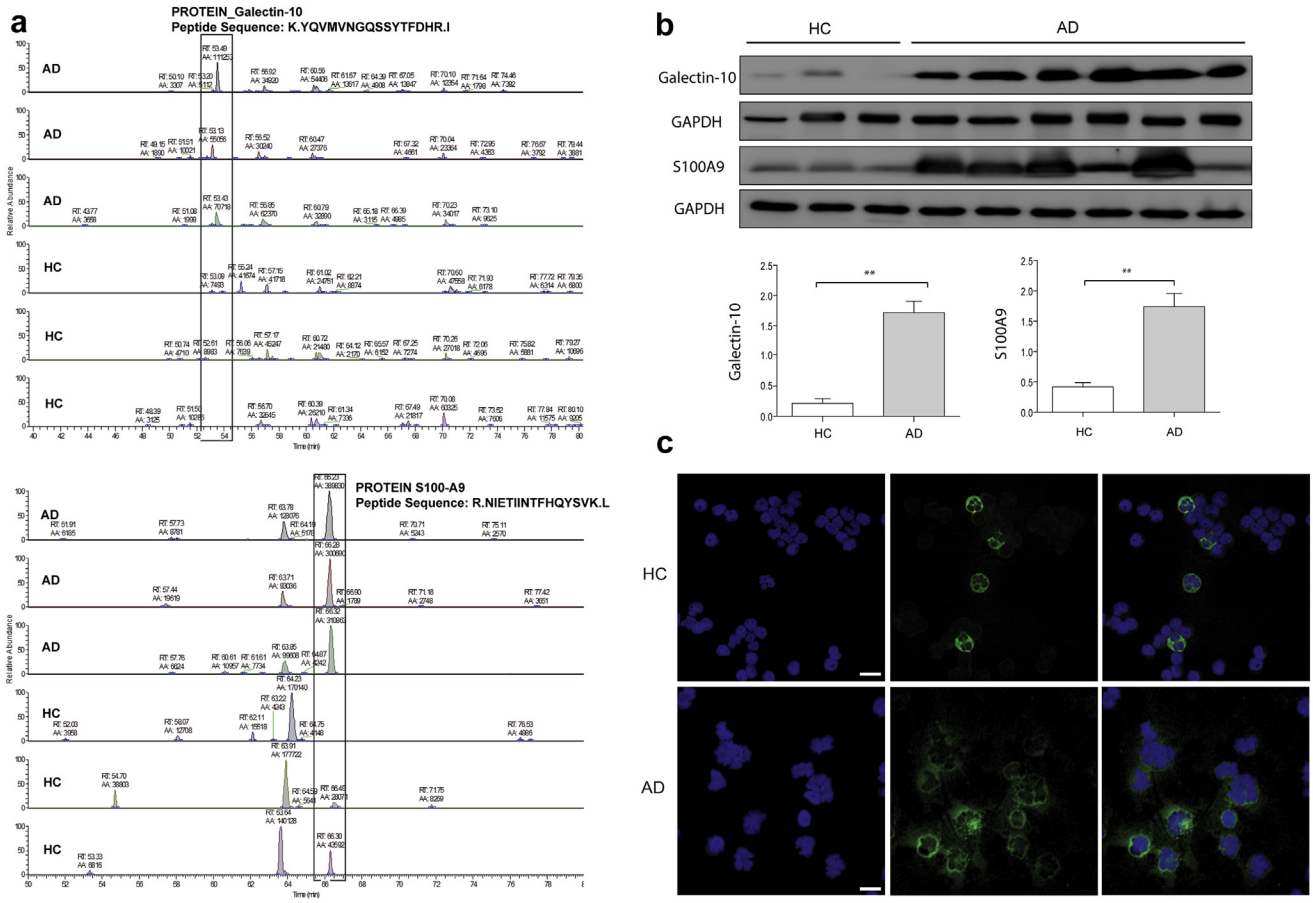

Figure 1. Proteomic analysis and western blot analysis of CD3$^+$ T cells from patients with AD and HCs. (**a**) Extracted ion chromatograms representing that peptides from galectin-10 and S100A9 were increased in T cells of patients with AD vs. HCs. (**b**) Western blot analysis of identified proteins in CD3$^+$ T cells from patients with AD and HCs. (**c**) Confocal laser microscopy of purified CD3$^+$ T cells stained with anti-galectin-10 (green) and DAPI (blue). Data shown are representative of three independent experiments. Scale bar =10 μm. $^{**}P < 0.01$. AD, atopic dermatitis; DAPI, 4′,6-diamidino-2-phenylindole; HCs, healthy controls.

serum IgE, eosinophil cationic protein, vitamin D, or eczema area. There was no significant difference of galectin-10 between extrinsic AD and intrinsic AD (data not shown).

In this study, we first performed large-scale analyses such as the normalized spectral index (SI_N) to identify differentially expressed proteins. As a local approach, we used extracted ion chromatography that determines abundance of specific target peptide converted from mass spectrometry-derived peptide data (Zybailov et al., 2005) to minimize errors caused by large-scale proteomics. Among S100A9 and galectin-10, galectin-10 was chosen for further analyses, as galectin-10 was recently shown to be associated with allergic diseases such as asthma.

Galectins, a family of animal lectins that bind *N*-acetyllactosamine-containing glycans deciphering the biological information of glycosylation signature, are known to have multiple roles in innate and adaptive immunity. For example, deletion of galectin-1 enhanced antigen-specific T_H1 and T_H17 responses and autoimmune inflammation (Blois et al., 2007; Toscano et al., 2007), whereas deletion of galectin-3 decreased pathogenic T_H1 and T_H17 responses reducing neuroinflammation (Jiang et al., 2009).

Among galectin family members, galectin-10 was initially described exclusively in eosinophils and basophils (Ackerman et al., 1982, 1993; Archer and Blackwood, 1965). However, CRTH2$^+$CD4$^+$ T_H2 memory cells were reported to undergo further T_H2 polarization and upregulated galectin-10, when activated by thymic stromal lymphopoietin-activated dendritic cells (Wang et al., 2006). Recently, mRNA of galectin-10 was reported to be overexpressed in the peripheral blood of patients with aspirin-induced asthma compared with aspirin-tolerant asthma (Devouassoux et al., 2008). Galectin-10 was reported to be expressed by human CD25$^+$ Treg cells and essential for their suppressive function (Kubach et al., 2007). In our study, galectin-10 was significantly increased in IL-22-producing T cells from peripheral blood and lesional skin of patients with AD compared with HCs. As we performed intracellular staining for flow cytometry without any activation, patients with AD present circulating activated T cells producing IL-22. As IL-22 causes epidermal hyperplasia, galectin-10 can be closely associated with epidermal acanthosis that is a hallmark of chronic AD lesion (Figure 2c). CD3$^+$ galectin-10$^+$ T cells from HCs showed the expression pattern of central memory T (T_{CM}, CD45-RO$^+$CCR7$^+$CD62L$^+$), not the patterns

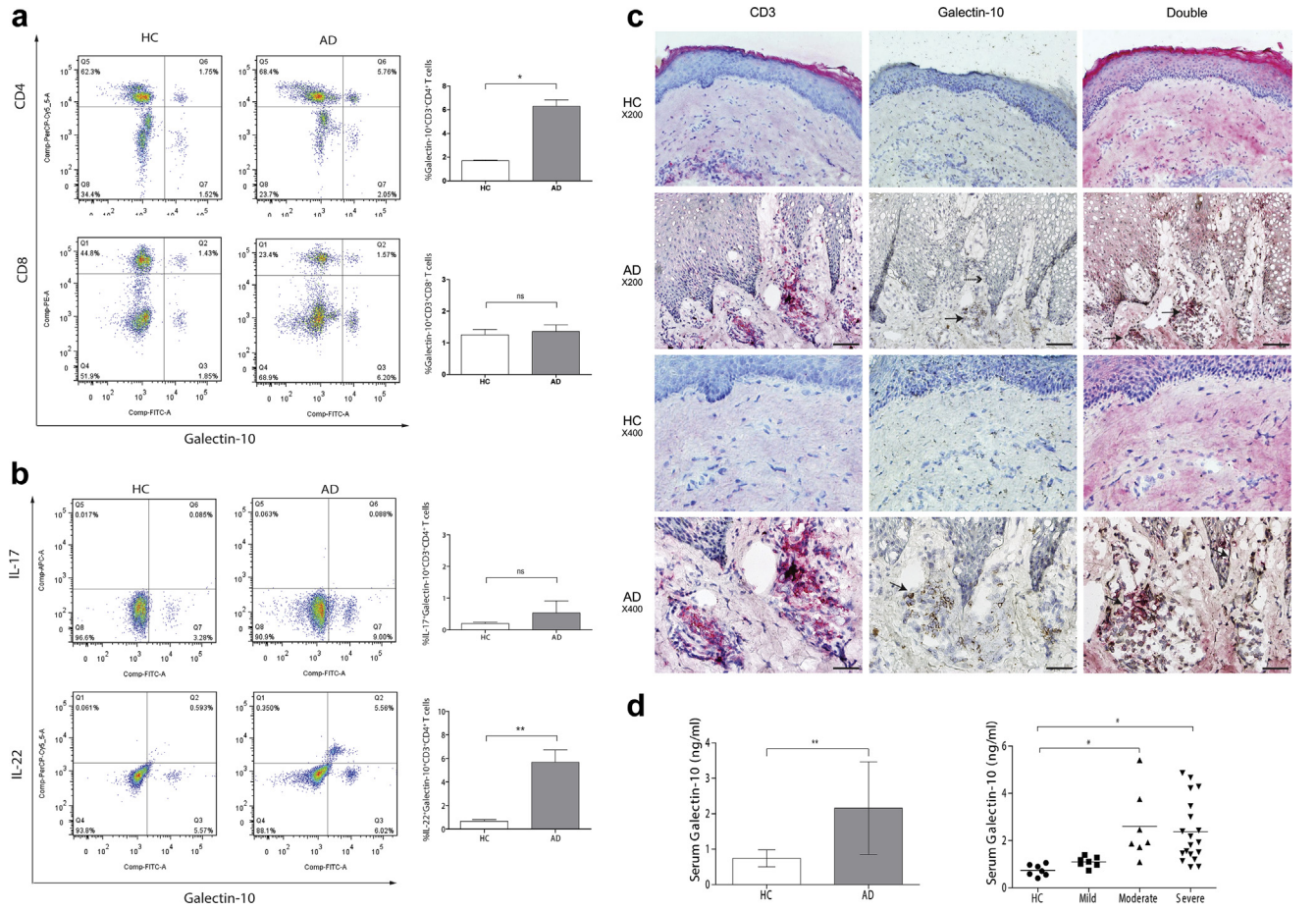

Figure 2. Increased expression of galectin-10 in IL-22-producing T cells, lesional skin, and sera of patients with AD. (**a**) Galectin-10 expression in $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells; the increase of galectin-10 in AD was mainly attributed to $CD3^+CD4^+$ T cells. (**b**) The expression of galectin-10 was significantly increased in IL-22-producing $CD3^+CD4^+$ T cells from patients with AD compared with HCs, whereas its expression was not significant in IL-17-producing $CD3^+CD4^+$ T cells. Data shown are representative of three independent experiments. (**c**) Galectin-10 expression in the skin of HCs and patients with AD. The superficial perivascular infiltrated $CD3^+$ T cells in AD were strongly stained with galectin-10 marked by an arrow with a straight line. Arrows with a dashed line indicate epidermal acanthosis adjacent to infiltrated $CD3^+$ galectin-10^+ T cells. Scale bars denotes for 100 μm (×200) and 50 μm (×400), respectively. (**d**) Serum levels of galectin-10 were increased in the moderate and severe patients with AD. $*P < 0.05$; $**P < 0.01$. AD, atopic dermatitis; HCs, healthy controls.

of naive or effector memory T (T_{EM}) cells (Supplementary Figure S3 online). As T_{CM} have skin homing properties, further experiments are required to study galectin-10^+ $CD3^+$ T cells in lesional skin (Clark et al., 2012).

The observations that the serum level of galectin-10 was increased in patients with AD compared with HCs and that these levels correlate positively with AD severity offer the possibility of galectin-10 as a disease biomarker. The origin of serum galectin-10 was not determined in this study. The observation that there is no significant correlation between eosinophil, eosinophil cationic protein, basophil, and lymphocyte count and serum galectin-10 level indicates that serum galectin-10 might be directly correlated with a certain subtype of T cells like T_H22 cells.

In summary, using proteomic analysis, we identified galectin-10 to be overexpressed in circulating $CD3^+$ T cells from patients with AD and in chronic AD skin compared with HCs. Increased expression of galectin-10 was also observed in IL-22-producing $CD4^+$ T cells from patients with AD. Serum levels of galectin-10 were higher in patients with AD relative to HCs, and this level correlated positively with AD severity. A further study of galectin-10^+ T cells in chronic AD is warranted to elucidate the clear role of galectin-10 in the pathogenesis of AD, and large-scale studies could reveal the possibility of galectin-10 as a disease biomarker for AD.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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Sub6 (Tri r 2), an Onychomycosis Marker Revealed by Proteomics Analysis of *Trichophyton rubrum* Secreted Proteins in Patient Nail Samples

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TO THE EDITOR

Onychomycosis, the most prevalent nail disease, is mainly caused by two dermatophyte species, *Trichophyton rubrum* and *Trichophyton interdigitale*, with a frequency in the range of 80% and 20%, respectively (Monod et al., 2002). To determine if the proteases secreted by *T. rubrum* in vitro during keratin digestion were involved in nail degradation, we investigated the fungus secretome in onychomycosis by proteomics analysis.

In a first experiment, mass spectrometry analyses were performed using a pool of extracts from 12 donors infected by *T. rubrum* as described in the Supplementary Materials and Methods online. Patient consent for experiments was not required because French laws consider human tissue leftover from surgery as discarded material. Proteins were extracted from each sample using a nonionic acid labile surfactant (ALS-400). The secretion of the following four secreted proteases of *T. rubrum* proteins extracted from nail beds was identified after subsequent SDS-PAGE separation and *in-gel* digestion coupled to mass spectrometry analysis: subtilisin-like protease 6 (Sub6, Q9UW97), subtilisin-like protease 7 (Sub7, Q8NID9), dipeptidyl-peptidase 5 (DppV, Q9UW98), and leucine aminopeptidase 2 (Lap2, Q5QHG6) (Table 1 and Supplementary Table S1 online). In particular, 12 unique peptides were found for Sub6, suggesting that this protease was abundantly secreted during nail infection. No *T. rubrum* proteins were detected in the collected samples from abnormal nails with trauma but without fungal infection (data not shown). No additional proteins were identified from a sequential second extract, suggesting that all soluble secreted proteins were already extracted in the first extraction (Supplementary Materials online). The high amount of Sub6 secreted by *T. rubrum* in onychomycosis and the presence of DppV were confirmed by Western blot analysis and by a shotgun protein identification experiment in SDS-PAGE gels using the same pooled extract for mass spectrometry analysis and specific antisera (Supplementary Table S2 and Figure S1 online). Surprisingly, most proteases secreted by the fungus during its in vitro growth in a keratin medium including subtilisin-like protease 3 (Sub3, B8XGQ6), subtilisin-like protease 4 (Sub4, A7UKV6), leucine aminopeptidase 1 (Lap1, Q5QHG5), dipeptidyl peptidase 4 (DppIV, Q5J6J3), and metallocarboxypeptidase (M14A, A6XGK3) (Giddey et al., 2007; Zaugg et al., 2008) were not detected either by mass spectrometry or by Western blot analysis (Supplementary Table S3 and Figure S1 online).

Abbreviations: MRM, multiple reaction monitoring

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