

FoxP3-expressing T regulatory cells in atopic dermatitis lesions

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ABSTRACT

Recently, studies were conducted to evaluate the impact of T regulatory (T regs) cells in the pathophysiology of atopic dermatitis (AD). The aim of this study was to investigate whether natural T regs are present in AD skin lesions. We performed skin biopsies in 12 adult patients affected by moderate-to-severe AD and 4 healthy volunteers. The specimens were stained immunohistochemically with anti-human CD25 and forkhead/winged helix transcription factor (FoxP3). Double immunostaining for CD25 and FoxP3 was performed also. CD25⁺ cells strongly infiltrated the perivascular and papillar dermis of all lesional specimens, and FoxP3⁺ cells were distributed in the perivascular and interstitial AD dermis, and some cells also infiltrated the dermoepidermal junction and the basal and suprabasal epidermal layers. All healthy skin specimens showed weak CD25 and FoxP3 stainings. Double immunostaining showed that CD25⁺ FoxP3⁺ cells were distributed in the perivascular, interstitial, and periadnexal dermis, and healthy skin specimens featured few CD25⁺ FoxP3⁺ cells scattered throughout the dermis. The past and present data show that an impaired function of natural T regs may not play a primary role in the pathophysiology of AD lesions.

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Key words: Atopic dermatitis, CD25, double immunostaining, FoxP3, immune regulation, immunohistochemistry, T regulatory cells

Atopic dermatitis (AD) is a chronic inflammatory skin disease associated with cutaneous hyperreactivity to environmental triggers.¹ The clinical phenotype that characterizes AD is the product of interactions between susceptibility genes, the environment, defective skin barrier function, and the dysregulation of the immune system.^{1,2}

T regulatory (T regs) cells play a key role in the modulation of allergic and autoimmune responses. Two main groups of T regs have been defined.³ One comprises the natural T regs, which are characterized by a CD4⁺ CD25⁺ phenotype and specifically express the forkhead/winged helix transcription factor (FoxP3), and the other group (so-called adaptive T regs) can be divided into T regs type 1, which secrete high levels of IL-10, and T helper type 3 cells, which

secrete high levels of transforming growth factor (TGF) β .

Recently, studies were conducted to evaluate the impact of T regs in the pathophysiology of AD.^{4–6} Ou *et al.* found that patients with AD had more CD4⁺ CD25⁺ T regs in peripheral blood than healthy control subjects and asthmatic patients.⁴ In contrast, other authors showed that peripheral T regs extracted from AD patients were numerically and functionally similar to those extracted from normal donors.⁵ Moreover, Verhagen *et al.* showed significant expression of IL-10 and TGF- β in AD lesional skin, and FoxP3 was not found despite the presence of many CD4⁺ CD25⁺ cells.⁶ The aim of this study was to further investigate whether natural T regs are present in chronic lesions of AD.

PATIENTS AND METHODS

Having obtained informed consent from 12 adult patients affected by moderate-to-severe AD, we performed a skin biopsy from chronic lesions of the trunk or limbs. Furthermore, also with informed consent, we obtained control specimens of normal-appearing skin from 10 volunteers and of normal human tonsils. Specimens, which had been immediately frozen at -80°C in liquid nitrogen, were cut into 5- μm -thick sections and

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stained immunohistochemically with antihuman CD25 (DAKO, Copenhagen, Denmark), anti-human FoxP3 ab10563 (Abcam, Ltd., Cambridge, U.K.), and polyclonal rabbit or mouse IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as isotype controls. FoxP3 peptide ab14151 (0.125 mg/mL; Abcam) was used as a control to block anti-FoxP3 binding.

Before staining, frozen sections were air-dried and fixed in acetone (5 minutes). Immunolabeling was performed with the alkaline phosphatase/anti-alkaline phosphatase method as described elsewhere.⁷ Negative control sections were incubated with nonimmune mouse sera.

To detect FoxP3-CD25⁺ cells, sections were subjected to horseradish peroxidase (HRP) staining of FoxP3 antigen, followed by alkaline phosphatase staining of CD25 antigen. Briefly, frozen sections of skin biopsy samples, fixed in cold acetone and dried, were allowed to come to room temperature (RT) and then were

rinsed in Tris-buffered saline plus 0.01% Tween-20 (TBS-T). Sections were then incubated with 5% normal goat serum (goat serum; Santa Cruz Biotechnology, Inc.) in phosphate-buffered saline (PBS) for 20 minutes at RT. To block endogenous biotin, sections were incubated with egg white in PBS for 30 minutes, washed with TBS-T, and then incubated with 5% skim milk in TBS-T for 30 minutes. Slides were then incubated overnight at 4°C with primary antisera for FoxP3 (mouse monoclonal antibody ab10563; Abcam, Ltd.) diluted at 1:40 in 5% normal goat serum in PBS. After three serial washes with TBS-T, sections were stained with an avidin-biotin peroxidase-based staining system according to the mouse ImmunoCruz Staining System (Santa Cruz Biotechnology, Inc.). Briefly, sections were incubated with biotinylated secondary antibody for 45 minutes at RT, and then rinsed with TBS-T. After blocking endogenous peroxidase activity by incubating specimens for 5 minutes in peroxidase block, sections

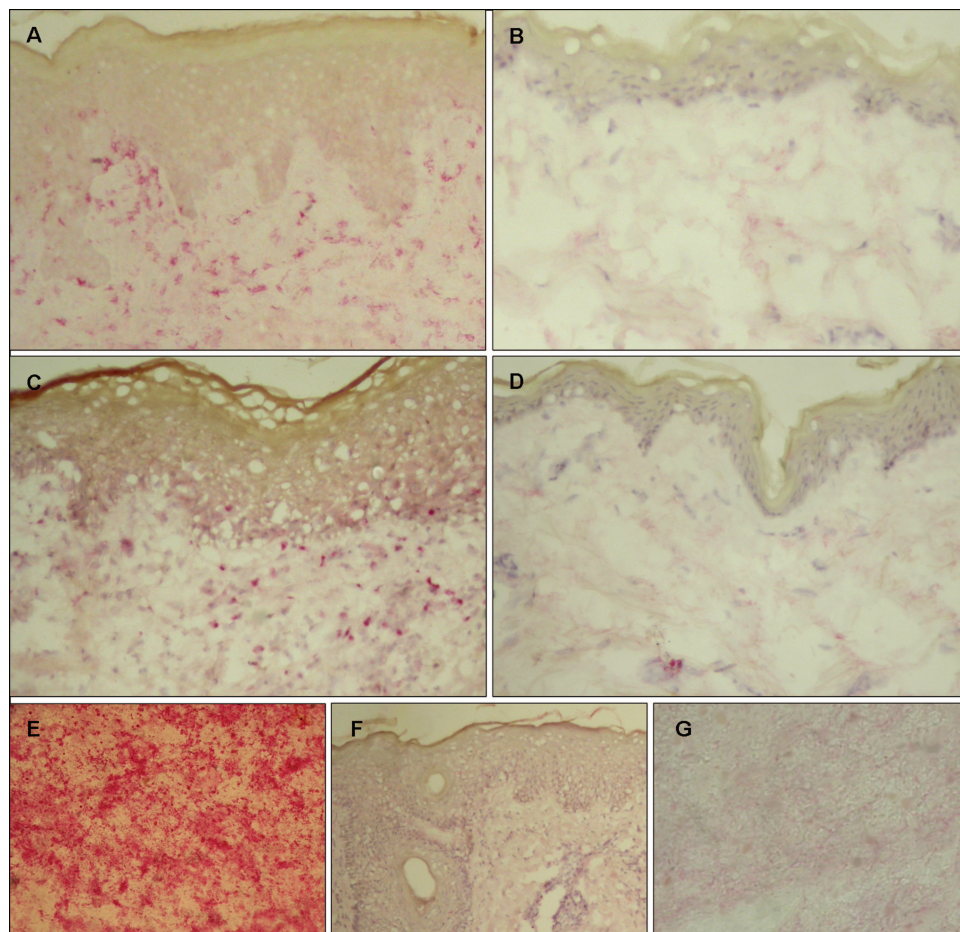


Figure 1. CD25 and FoxP3 stainings in AD skin and controls (original magnification, $\times 200$). (A) CD25⁺ cells strongly infiltrate the lesional dermis until the dermoepidermal junction; mild exocytosis also is appreciable in the deeper epidermal layers. (B) CD25⁺ cells scattered in the healthy skin perivascular dermis. (C) Perivascular, interstitial, and junctional distribution of FoxP3-expressing cells in an atopic dermatitis lesion. Some positive cells also infiltrate the epidermis. (D) Blockage of FoxP3 staining in AD skin by preincubating with FoxP3 peptide. (E) Healthy skin shows rare FoxP3⁺ dermal cells. (F) FoxP3⁺ cells within a healthy tonsil specimen. (G) Blockage of FoxP3 staining in a healthy tonsil with specific peptide.

Table 1 Means $\pm$ SD and statistical comparisons between AD and HC specimens

	AD	HC	<i>p</i> Value
FoxP3 ⁺	20.04 $\pm$ 7.91	5.34 $\pm$ 2.21	<0.0001
CD25 ⁺	36.41 $\pm$ 15.38	6.54 $\pm$ 4.12	<0.0001
FoxP3 ⁺ CD25 ⁺	18.82 $\pm$ 8.45	4.43 $\pm$ 3.39	<0.0001

HC = healthy skin controls.

were incubated for 30 minutes at RT in HRP–streptavidin complex. After, two serial washes with TBS-T, sections were incubated with HRP substrate and developed until light brown. Then, the sections were rinsed with deionized H₂O and transferred to TBS-T. Next, sections were incubated overnight at 4°C with second primary antiserum for CD25 (rabbit polyclonal antibody N-19, used at 1:40; Santa Cruz Biotechnology, Inc.). Sections then were rinsed with TBS-T and incubated with alkaline phosphatase–conjugated secondary antibody (bovine anti-goat IgG-AP or bovine anti-rabbit IgG-AP [Santa Cruz Biotechnology, Inc.], used at 1:200 dilution in PBS) for 45 minutes at RT. Then, sections were washed with TBS-T and incubated for 10

minutes at RT with levamisole to inhibit endogenous alkaline phosphatase activity. Next, sections were stained with an alkaline phosphatase substrate kit until light blue (Vector Blue Substrate Kit; Vector Laboratories, Ltd., Peterborough, England) and mounted in an aqueous-based mounting medium. Negative controls were included with each run by substituting the primary antibodies with no immune sera.

Two independent “blind” observers evaluated serial sections. For quantitative analysis, the stained cells were counted in three consecutive microscopic fields (250 $\times$). Statistical analysis was performed with a Wilcoxon rank sum test for comparing AD group with

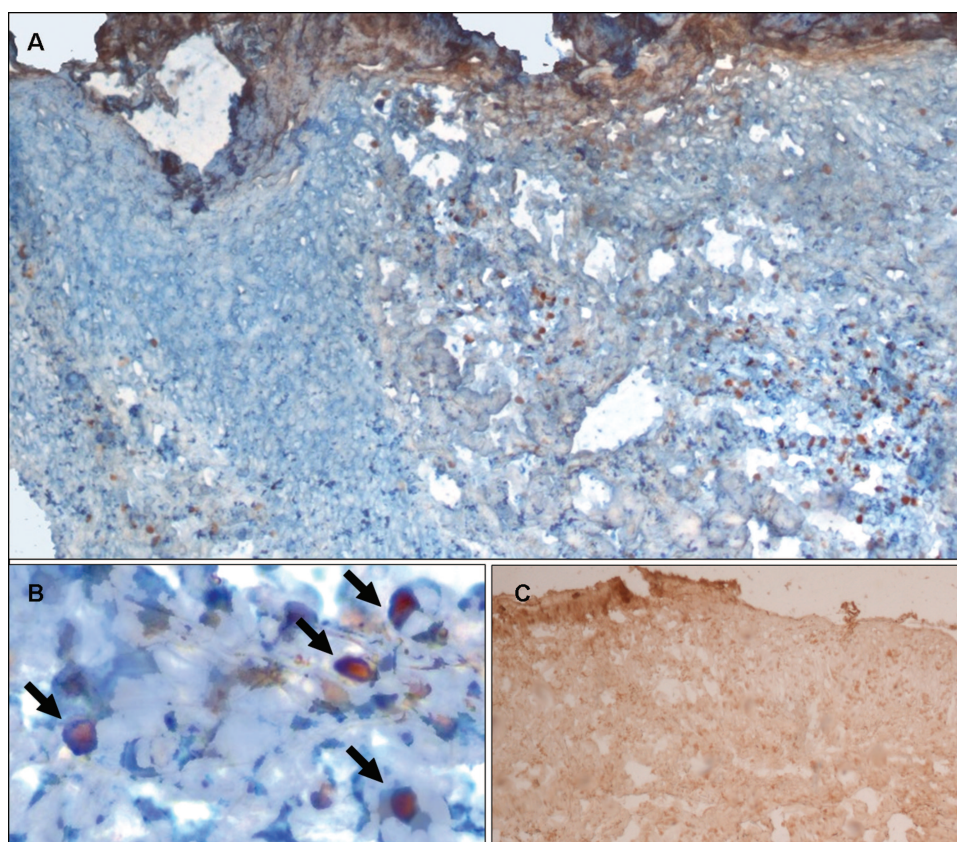


Figure 2. CD25/FoxP3 double immunostaining in AD skin. (A) CD25⁺ FoxP3⁺ cells are mainly distributed in the perivascular and periadnexal dermis (original magnification, $\times 200$). (B) Higher magnification photomicrograph showing some double stained cells. FoxP3 staining (light brown) is localized within the nucleus, and anti-CD25 stains the peripheral cell portion in light blue (original magnification, $\times 300$). (C) No staining is detectable in a negative control. Arrows indicate double-positive cells (original magnification, $\times 200$).

controls. The value of $p < 0.05$ was considered statistically significant.

RESULTS

CD25⁺ cells strongly infiltrated the perivascular and papillary dermis of all lesional specimens (Fig. 1 A), and only a few positive cells featured the healthy control skin (Fig. 1 B; Table 1). FoxP3-expressing cells were distributed in the perivascular and interstitial AD dermis, some cells also infiltrating the dermoepidermal junction and the basal and suprabasal epidermal layers (Fig. 1 C). All healthy skin specimens showed a weak FoxP3 staining (Fig. 1 D), and FoxP3⁺ cells were detectable abundantly in the tonsils (Fig. 1 E). The binding of FoxP3 antibody could be blocked by preincubating the antibody with specific FoxP3 peptide both in AD skin (Fig. 1 F) and in tonsils (Fig. 1 G).

Double immunostaining showed that CD25⁺ FoxP3⁺ cells were distributed in the perivascular, interstitial, and periadnexal dermis (Fig. 2, A–B). Healthy skin specimens featured few CD25⁺ FoxP3⁺ cells scattered throughout the dermis. Negative controls showed no specific staining (Fig. 2 C).

DISCUSSION

Currently, it is suggested that the development of allergy may be caused by insufficient development of allergen-specific T regs in atopic individuals and that such T regs deficiency would progressively decrease from acute to chronic phases of disease. Recently, Verhagen *et al.* showed that FoxP3⁺ cells are absent in cutaneous lesions of AD.⁶ By contrast, our results showed the presence of CD25⁺ FoxP3⁺ T regs in chronic AD lesions. The discrepancy between our observation and that by Verhagen *et al.*⁶ possibly lies in the different evolutive phase of the lesions studied, although such authors did not specify the clinical features of their biopsied lesions. Moreover, our findings agree with recent studies, which showed that CD4⁺ CD25⁺ cells are present in the peripheral blood of AD patients^{4,5} and that several of those cells express FoxP3.⁴

Although our results showed that natural T regs are present in chronic AD lesions, there is some evidence suggesting a lack in the immune response regulation mediated by adaptive T regs in AD patients. In fact, although a recent study showed an increase of TGF- β expression in AD lesional skin,⁶ subnormal levels of TGF- β seemed to strongly correlate with AD.^{8,9} Ac-

cordingly, we recently highlighted a reduced TGF- β staining in chronic AD lesions in comparison with healthy controls.⁷ Interestingly, a short-term therapy with tacrolimus ointment, an effective and well-tolerated agent for AD,^{10,11} achieved a clinical improvement of the lesions and increased the pool of TGF- β ⁺ cells, until reaching the levels observed in the normal skin.⁷ Instead, FoxP3⁺ cells were not affected by a treatment course with tacrolimus and hydrocortisone butyrate; *i.e.*, their number remained almost unchanged between untreated lesions and healed skin.⁷

To conclude, the present data show that natural T regs are present in chronic AD lesions. Additional studies may allow a better understanding of the putative role of such cells in the pathophysiology of AD.

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