

Analysis of CD25^{hi}CD4⁺ “regulatory” T-cell subtypes in atopic dermatitis reveals a novel T_H2-like population

Amanda J. Reefer, MS,^a Shama M. Satinover, MS,^a Michael D. Solga, MS,^b Joanne A. Lannigan, MS,^b Jennifer T. Nguyen, BS,^a Barbara B. Wilson, MD,^c and Judith A. Woodfolk, MBChB, PhD^a Charlottesville, Va

Background: It is unresolved whether circulating CD25^{hi}CD4⁺ T cells in patients with atopic dermatitis who have elevated IgE (IgE^{high}) are regulatory or effector in nature.

Objective: To analyze the properties of CD25^{hi} T-cell subtypes in IgE^{high} atopic dermatitis.

Methods: The phenotype of circulating CD25^{hi} T cells was analyzed by flow cytometry using PBMCs from patients with atopic dermatitis (total IgE > 250 IU/mL). Cytokines induced in CD25^{hi} subtypes were analyzed after activation with anti-CD3 mAb (±IL-2) and in the presence of activated autologous effector T cells (CD25^{neg}CD4⁺). Reactivity to bacterial superantigen derived from the skin-colonizing organism *Staphylococcus aureus* was also evaluated.

Results: CD25^{hi} T cells expressing regulatory T-cell markers (Foxp3, CCR4, cutaneous lymphocyte-associated antigen) were increased in atopic dermatitis compared with IgE^{low} controls. This phenomenon was linked to disease severity. Two subtypes of CD25^{hi} T cells were identified on the basis of differential expression of the chemokine receptor CCR6. Although the ratio of CCR6⁺ and CCR6^{neg} subtypes within the CD25^{hi} subset was unaltered in atopic dermatitis, each subtype proliferated spontaneously *ex vivo*, suggesting *in vivo* activation. Activated CCR6^{neg} cells secreted T_H2 cytokines, and coculture with effector T cells selectively enhanced IL-5 production. Moreover, induction of a T_H2-dominated cytokine profile on activation with bacterial superantigen was restricted to the CCR6^{neg} subtype.

Conclusion: Despite a regulatory phenotype, activated CD25^{hi} T cells that lack expression of CCR6 promote T_H2 responses. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Human regulatory T cells, T_H2 cytokines, CCR4, CLA, CCR6, CD25, Foxp3, bacterial superantigen, allergen avoidance

Abbreviations used

AD:	Atopic dermatitis
APC:	Antigen-presenting cell
CLA:	Cutaneous lymphocyte-associated antigen
GITR:	Glucocorticoid-induced TNF receptor-related protein
SCORAD:	SCORing Atopic Dermatitis
SEB:	Staphylococcal enterotoxin B

The presence of very high levels of serum IgE and allergen-specific IgE antibodies in a subset of patients with the inflammatory skin condition atopic dermatitis (AD) has been attributed to T_H2 skewing in response to allergens and other stimuli.¹⁻⁵ There is mounting evidence of a role for CD25⁺CD4⁺ regulatory T cells in suppressing T-cell responses to allergens⁶⁻¹⁰; however, little is known about these cells in patients with AD. Studies on circulating CD25⁺CD4⁺ T cells are challenging in healthy human beings without allergy because of variable expression of CD25 and restriction of suppressor function to a small minority of cells that express the highest levels of CD25.¹¹⁻¹³ In addition to their low frequency, CD25^{hi}CD4⁺ T cells with suppressive capacity have been difficult to isolate because of the absence of a reliable surface marker. Expression of the transcription factor, Foxp3, is critical to the development of CD25^{hi}CD4⁺ regulatory T cells with suppressor function, and this has provided a useful marker for regulatory T cells in healthy individuals. However, in human beings, Foxp3 expression can be induced in activated effector T cells, and these cells also express CD25.¹⁴⁻¹⁶ Thus, human CD25^{hi}CD4⁺ T cells associated with inflammatory diseases such as AD may be a mixture of activated effector T cells and regulatory T cells, which could lead to confounding observations in experimental systems.

In healthy individuals, the majority of CD25^{hi}Foxp3⁺ regulatory T cells in the blood express an array of functional skin-homing receptors (cutaneous lymphocyte-associated antigen [CLA], CCR4, and CCR6). These cells are present in the skin and possibly fulfill an important role in immune surveillance at this site.^{12,13} An increase in circulating CD25⁺CD4⁺ T cells has been described in AD,¹⁷ which could reflect a breakdown in the cutaneous immune surveillance system. The current study was designed to examine the properties of CD25^{hi}CD4⁺ T cells that express Foxp3 in the blood of patients with AD with a view to assessing regulatory versus effector properties. We describe a novel CD25^{hi} T-cell subtype with T_H2-like properties that lacks expression of the chemokine receptor, CCR6. Our results highlight the complexity of regulatory T cells in human beings and have important implications for existing T-cell paradigms related to allergic disease.

From ^athe Asthma and Allergic Diseases Center; ^bthe Flow Cytometry Core Facility, Department of Microbiology; and ^cthe Department of Dermatology, University of Virginia Health System.

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Reprint requests: Judith A. Woodfolk, MBChB, PhD, Allergy Division, PO Box 801355, University of Virginia Health System, Charlottesville, VA 22908-1355. E-mail: jaw4m@virginia.edu.

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TABLE I. Characteristics of IgE^{high} patients with AD and IgE^{low} subjects without AD

IgE ^{high} (AD)				IgE ^{low} (non-AD)	
Patient no. (age/sex/race)	SCORAD index	Total IgE (IU/mL)*		Patient no. (age/sex/race)	Total IgE (IU/mL)
1† (40/M/W)	78 (Severe) [60 (Severe)]	16149 [21,230]		15 (37/M/W)	134
2 (45/F/B)	53 (Severe)	13,006		16 (24/F/W)	113
3 (35/F/W)	37 (Moderate)	10,638		17 (37/F/W)	62
4† (37/F/B)	50 (Severe) [90 (Severe)]	6165 [4195]		18 (25/M/W)	45
5† (24/F/W)	33 (Moderate) [67 (Severe)]	3417 [4761]		19 (30/F/W)	41
6 (41/F/W)	12 (Mild)	3326		20 (43/F/W)	28
7 (23/F/W)	45 (Severe)	1965		21 (37/F/W)	28
8 (39/F/W)	44 (Severe)	1911		22 (29/M/W)	21
9 (21/M/W)	32 (Moderate)	1591		23 (27/F/W)	16
10 (19/M/W)	30 (Moderate)	1232		24 (25/F/W)	15
11 (53/F/B)	40 (Moderate)	1121		25 (24/F/W)	12
12 (20/F/W)	51 (Severe)	441		26 (46/F/W)	11
13 (40/F/W)	24 (Moderate)	289		27 (36/F/W)	5
14 (13/M/W)	80 (Severe)	26,322		28 (29/F/W)	5

M, Male; W, white; F, female; B, black.

*Quantitation of IgE was by CAP assay.

†Values in parentheses correspond to baseline measures for allergen avoidance study.

METHODS

Subjects

Patients with AD were diagnosed by using the criteria of Hanifin and Rajka.¹⁸ T-cell studies were performed in 14 patients with AD (13 adults ages 19–53 years and 1 child, age 13 years) with high total IgE (≥ 250 IU/mL) and a clinical history of early disease onset (age < 1 year). Severity of disease was assessed by the Scoring Atopic Dermatitis (SCORAD) index.¹⁹ Healthy subjects without AD (ages 24–46 years; n = 14) who had low total IgE (<140 IU/mL) and reported no allergic symptoms were IgE^{low} controls. No patients received systemic immunosuppressive drugs within 6 months of starting T-cell studies. Studies on human subjects were approved by the University of Virginia Human Investigation Committee.

Antibodies and reagents

See this article's [Methods](#) section in the Online Repository at www.jacionline.org.

Analysis of cell surface antigens and intracellular Foxp3 protein

PBMCs were stained with fluorochrome-conjugated mAbs and analyzed by flow cytometry immediately after isolation. Expression of Foxp3 protein was performed in conjunction with staining for surface antigens by using the Foxp3 staining set (clone PCH101; eBioscience, San Diego, Calif). See this article's [Methods](#) section in the Online Repository for details on flow cytometry analysis.

Proliferation assays and cytokine detection

CD4⁺ T cells were isolated by magnetic activated cell sorting (Miltenyi Biotec, Auburn, Calif), stained with anti-CD4–fluorescein isothiocyanate, anti-CD25–phycoerythrin, anti-CCR4–phycoerythrin-Cy7, and anti-CCR6–allophycocyanin (mAbs (BD Biosciences, Franklin Lakes, NJ), and sorted by flow cytometry (BD FACS Vantage SE Turbo sorter). CD25^{hi}CCR6⁺ and CD25^{hi}CCR6^{neg} subtypes were sorted from gated CD25^{hi}CCR4⁺ T cells representing ~3% of total CD4⁺ T cells expressing the highest levels of CD25. T-cell reactivity was assessed by culturing sorted populations (1.5×10^4 per well in triplicate) in 96-well flat-bottom plates coated with antihuman CD3 mAb (BD Biosciences) $\pm$ IL-2 (100 U/mL). Proliferation was measured on day 5 by [³H] thymidine incorporation (Topcount NXT; Packard Bioscience Co, Downers Grove, Ill), and cytokines were measured by cytometric bead assay (T_H1/T_H2 panel, Bio-Plex System; BioRad, Hercules, Calif). In other experiments, sorted T-cell populations (10^4 per well; 3–6 replicates) were stimulated

with 20 ng/mL staphylococcal enterotoxin B (SEB) (Sigma, St Louis, Mo) in the presence of irradiated CD14⁺ monocytes and cytokines, and proliferation was measured on day 3. For coculture experiments, sorted whole CD25^{hi} T cells or CD25^{hi}CCR6⁺ or CD25^{hi}CCR6^{neg} cells (2.5×10^4 per well) were cultured with CD25^{neg}CD4⁺ autologous effector T cells (2.5×10^4 per well) in the presence of irradiated monocytes and plate-bound anti-CD3 mAb. Cytokines were measured at 24-hour intervals (days 1–5) in harvested supernatants pooled from 3 to 6 replicate wells, and proliferation was measured on day 5.

Allergen avoidance trial

Three patients with severe AD and elevated IgE (total IgE > 3000 IU/mL and multiple sensitivities to allergen) were admitted to the University of Virginia General Clinical Research Center. Patients lived in an allergen-free hospital room for 2 (patient 5) or 3 (patients 1 and 4) weeks and received dietary restriction on the basis of food sensitivities. Patients received no pharmacologic intervention. PBMCs were analyzed before (day 0) and after (days 14 or 21) allergen avoidance.

Statistical analysis

See this article's [Methods](#) section in the Online Repository.

RESULTS

Increased circulating CD25^{hi}CD4⁺ T cells in patients with AD exhibit a surface phenotype consistent with skin-homing regulatory T cells

The properties of circulating CD25^{hi}CD4⁺ T cells were compared in IgE^{high} patients with AD and IgE^{low} healthy controls ([Table I](#)). By using 7-color flow cytometry staining, we analyzed tissue-homing receptors normally expressed on CD25^{hi} regulatory T cells in healthy individuals.^{12,13,20–22} Almost all CD4⁺ T cells that expressed the highest levels of CD25 also expressed the chemokine receptor CCR4 regardless of disease status ([Fig 1, A](#); see this article's [Fig E1](#) in the Online Repository at www.jacionline.org). Thus, to perform a stringent analysis of CD25^{hi} T cells, we gated on CD25^{hi}CCR4⁺ T cells and compared these cells with 4 other populations that differentially expressed CCR4 and CD25: (1) CD25^{mid}CCR4⁺; (2) CD25^{neg}CCR4⁺; (3) CD25⁺CCR4^{neg}; and (4) CD25^{neg}CCR4^{neg} ([Fig 1, A](#); [Fig E1](#)). The frequency of CD25^{hi}CCR4⁺ T cells (hereafter referred to

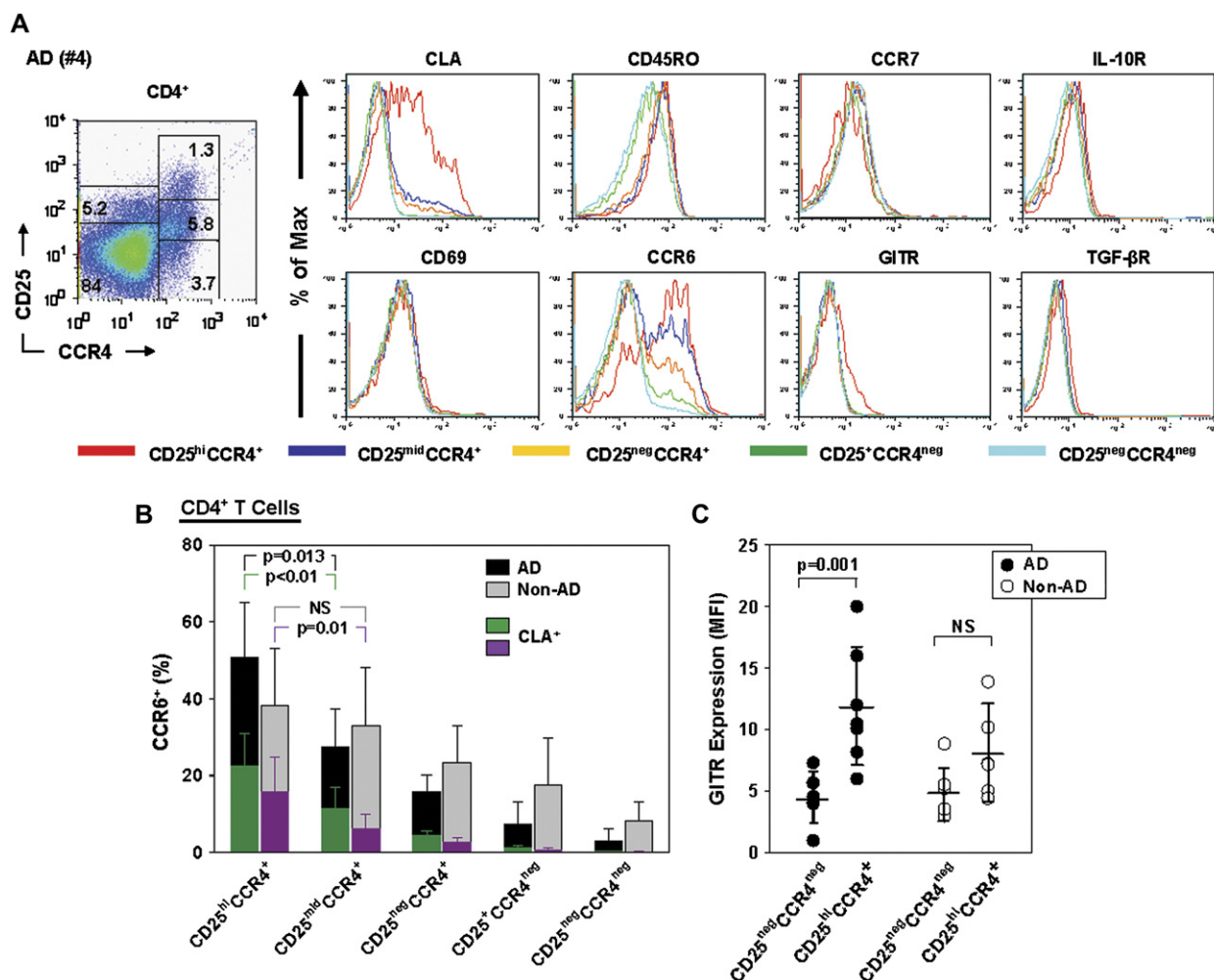


FIG 1. Surface phenotype of CD25^{hi} T cells in AD. **A**, Expression of surface markers on CD25^{hi}CCR4⁺ T cells compared with other CD4⁺ T cell subsets that differentially express CD25 and CCR4 (see *gates* in *dot plot*). Values in *dot plot* indicate cell percentages (representative of 6 patients with AD). *IL-10R*, IL-10 receptor; *TGF-βR*, TGF-β receptor. **B**, Percentage of CCR6⁺ (black and grey) and CCR6⁺CLA⁺ (color) cells within each subset (n = 6 per group; *P < .03). Error bars represent SDs. **C**, Mean fluorescence intensity of GITR expression on CD25^{hi}CCR4⁺ and CD25^{neg}CCR4^{neg} T cells (AD, n = 7; non-AD, n = 6). NS, Not significant.

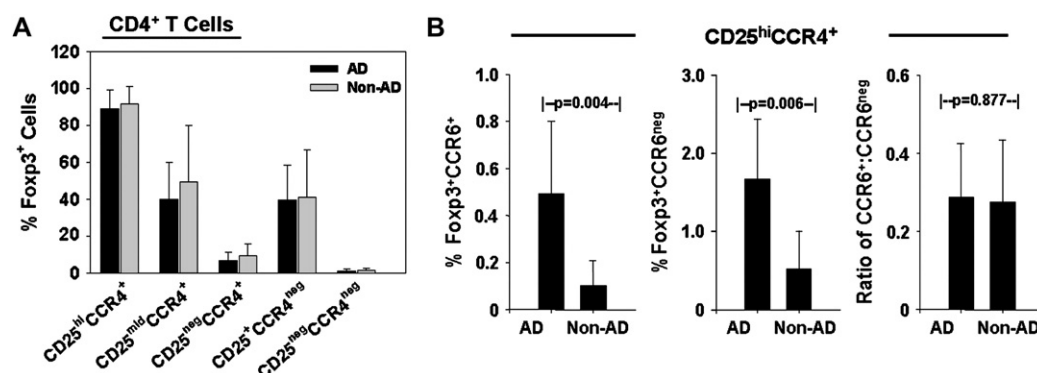


FIG 2. Foxp3 expression in CD25^{hi} T cells. **A**, Percentage of Foxp3⁺ T cells in CD4⁺ T-cell subsets that differentially express CD25 and CCR4 (n = 7 per group). **B**, Frequency of Foxp3⁺CCR6⁺ and Foxp3⁺CCR6^{neg} cells within the CD25^{hi} subset expressed as a percentage of total CD4⁺ T cells (n = 7 per group). Error bars represent SDs.

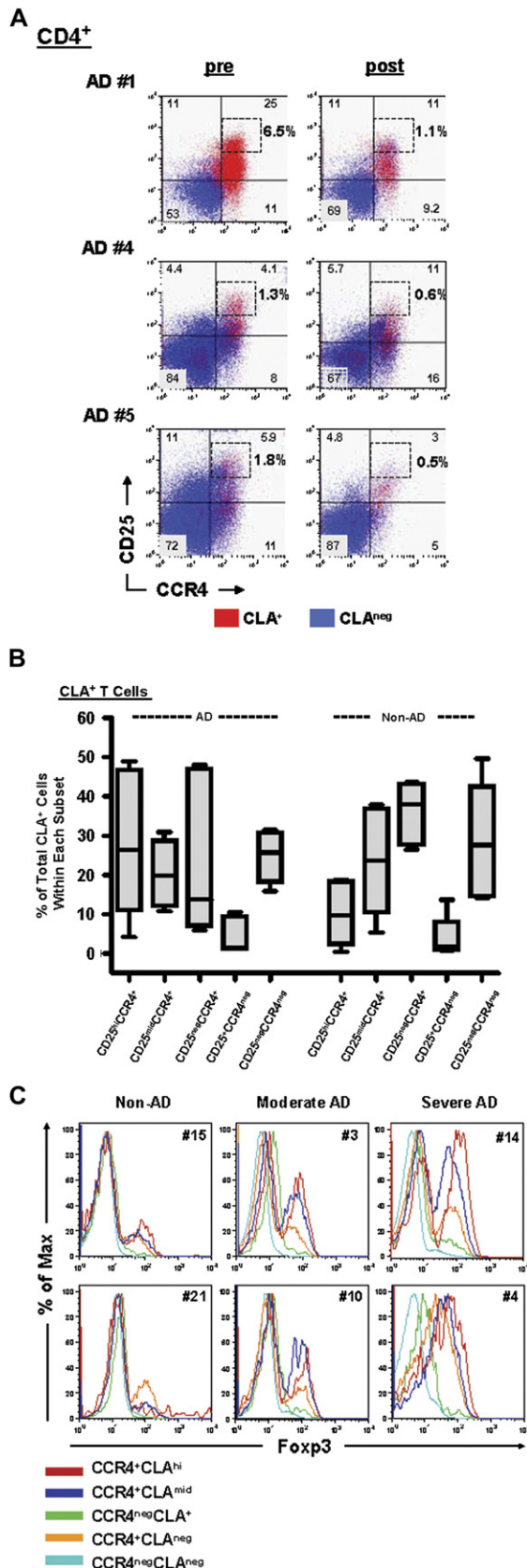


FIG 3. CD25^{hi} T cells and disease severity. **A**, Expression of CD25 and CCR4 on CD4⁺ T cells before (*pre*) and after (*post*) allergen avoidance. Values are percentages of cells in each quadrant and in the CD25^{hi}CCR4⁺ subset

as CD25^{hi} T cells) was significantly increased in patients with AD ($2.4\% \pm 1.4\%$ of total CD4⁺ T cells) compared with subjects without AD ($0.95\% \pm 0.5\%$; $P = .04$). CD25^{hi} T cells expressed CD45RO and low levels of CCR7 in both patients with AD and subjects without AD (Fig 1, A; Fig E1). This phenotype is characteristic of effector memory T cells, which traffic preferentially from blood into tissues.²³ Similar to subjects without AD, CD25^{hi} T cells from patients with AD expressed high levels of CLA (mean, 55%; range, 38% to 76%; Fig 1, A; Fig E1; and data not shown) as well as high levels of CCR6 (Fig 1, B). Moreover, the percentage of cells coexpressing CLA and CCR6 was highest in the CD25^{hi} subset (Fig 1, B). Expression of receptors for the regulatory cytokines IL-10 and TGF- β was not a feature of CD25^{hi} T cells in either group (Fig 1, A; Fig E1). Finally, we examined expression of the inhibitory signaling molecule, glucocorticoid-induced TNF receptor-related protein (GITR). This molecule is upregulated in activated T cells and has been implicated in the altered function of regulatory T cells in AD.²⁴ Although GITR expression was increased on CD25^{hi} T cells compared with CD25^{neg}CCR4^{neg} cells in patients with AD and subjects without AD, significant upregulation was restricted to the AD group (Fig 1, C). Thus, CD25^{hi} T cells in the blood of patients with AD exhibit a cell surface phenotype typical of regulatory T cells from normal individuals. Increased GITR expression may reflect activation of these cells *in vivo*.

Both CD25^{hi}CCR6⁺ and CD25^{hi}CCR6^{neg} T cells expressing Foxp3 are increased in AD

Although CCR6⁺ cells were enriched within the CD25^{hi} subset, a large proportion ($\sim 50\%$) of CD25^{hi} cells were CCR6^{neg}. We postulated that these CCR6^{neg} cells may constitute a distinct subtype. To examine this further, we examined whether expression of Foxp3 protein differed between CCR6^{neg} and CCR6⁺ subtypes. The vast majority of CD25^{hi} cells in both AD ($89\% \pm 10.2\%$) and non-AD ($91\% \pm 9.6\%$) groups expressed Foxp3 (Fig 2, A) with no difference between CCR6⁺ and CCR6^{neg} subtypes. Consistent with the increase in total CD25^{hi} T cells in AD, the frequency of both Foxp3⁺CCR6⁺ and Foxp3⁺CCR6^{neg} subtypes (as a percentage of total CD4⁺ T cells) was increased in AD ($P < .01$); however, the ratio of these subtypes within the CD25^{hi} subset was unchanged, suggesting that homeostasis of these cell populations is maintained (Fig 2, B).

Circulating CD25^{hi} Foxp3⁺ T cells with skin-homing potential are linked to disease severity

In patients with AD, the majority of CD4⁺ T cells with skin-homing potential (ie, those that coexpressed CCR4 and CLA) were CD25^{hi} T cells (Fig 3, A, left panel). Furthermore, a higher proportion of total CLA⁺ T cells were CD25^{hi} in AD compared with non-AD (Fig 3, B). Given that CD25^{hi} T cells were increased in AD and the majority of these cells were Foxp3⁺, we predicted that Foxp3⁺ cells would be increased in skin-homing T cells from patients with AD, as was the case (Fig 3, C). Moreover, the proportion of Foxp3⁺ cells in skin-homing T cells was highest in

(hatched box). **B**, Distribution of total CLA⁺ T cells among different CD4⁺ T-cell subsets (medians and interquartile ranges, $n = 6$ per group). **C**, Expression of Foxp3 in CD4⁺ T-cell subsets that differentially express CLA and CCR4 for 6 patients with different disease status.

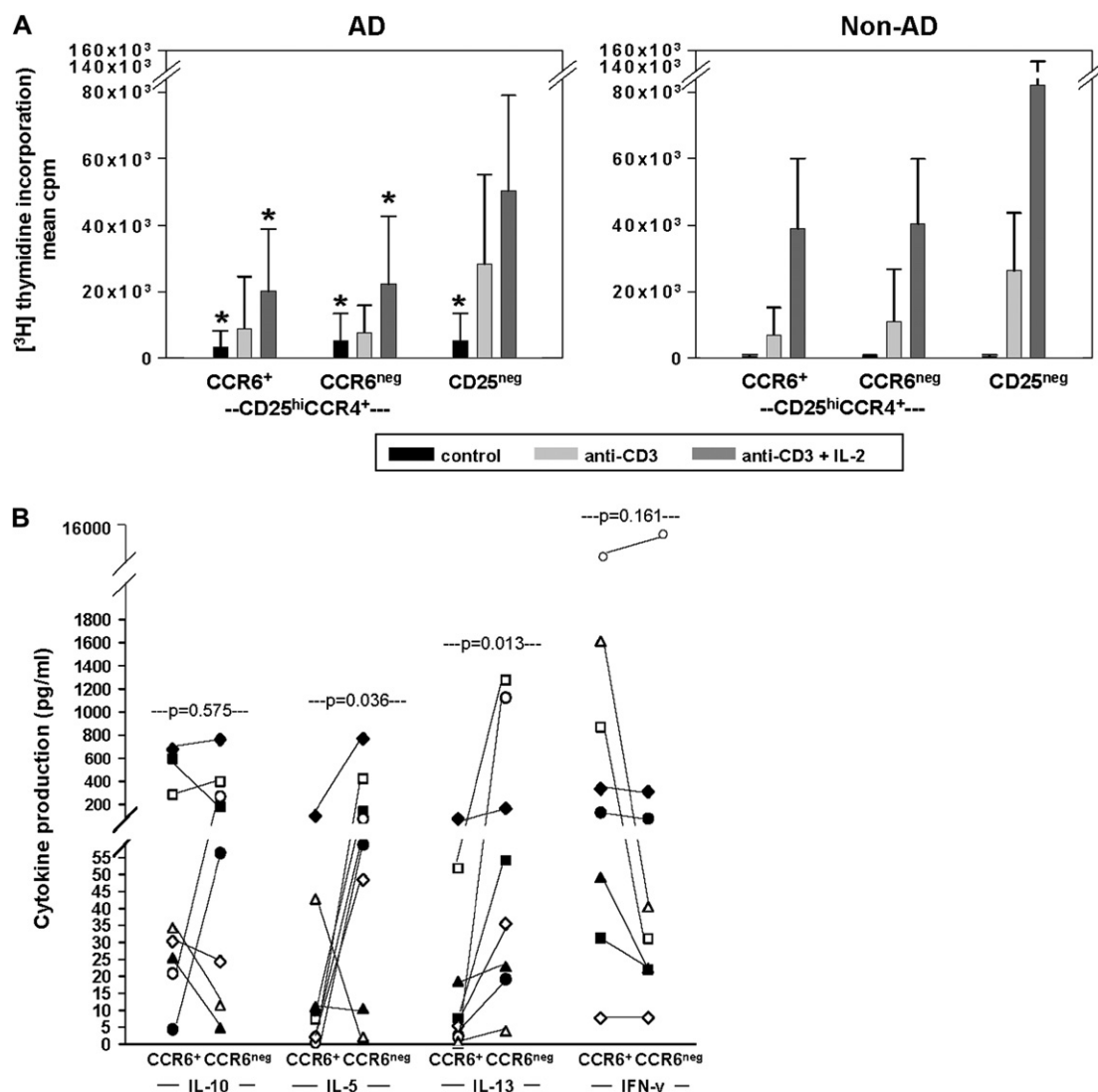


FIG 4. Proliferation and cytokine responses of CD25^{hi} subtypes. **A**, Proliferation of CD25^{hi}CCR6⁺ or CD25^{hi}CCR6^{neg} subtypes and CD25^{neg} CD4⁺ T cells in response to different T-cell stimuli (day 5; n = 5 per group). **P* < .05 for AD vs non-AD. Error bars represent SDs. cpm, Counts per minute. **B**, Cytokine levels (day 5) after stimulation of sorted CD25^{hi} subtypes with anti-CD3 mAb + IL-2 (AD [open symbols] and non-AD [closed symbols], n = 4 per group).

severe disease (Fig 3, C). Thus, we next examined the link between CD25^{hi} T cells and disease status. To this end, changes in circulating CD25^{hi} T cells were monitored in 3 patients with severe disease who received a complete allergen avoidance regimen (Table I). After allergen avoidance, the frequency of CD25^{hi} T cells, including CLA⁺ cells, decreased 2-fold to 6-fold (Fig 3, A) in parallel with marked improvement in skin condition (SCORAD for preavoidance and postavoidance: patient 1, 60 vs 35; patient 4, 90 vs 42; patient 5, 67 vs 31). These findings support a link between disease severity and circulating CD25^{hi} Foxp3⁺ T cells with skin-homing capacity.

The CD25^{hi}CCR6^{neg} subtype exhibits T_H2-promoting properties

Because circulating CCR6⁺ and CCR6^{neg} subtypes that expressed high levels of CD25 and Foxp3 were increased in patients

with AD, we examined whether these cells exhibited functional properties of regulatory T cells *in vitro*. CD25^{hi} subtypes were purified (>99% purity) by flow cytometry sorting and their proliferative capacity and cytokine profiles analyzed *ex vivo*. Unlike conventional regulatory T cells, both CCR6⁺ and CCR6^{neg} subtypes from patients with AD proliferated spontaneously, consistent with *in vivo* activation, whereas those from subjects without AD did not (CCR6⁺: 3375 ± 4860 cpm vs 443 ± 660 cpm [*P* = .042]; CCR6^{neg}: 5312 ± 8067 vs 482 ± 572 cpm [*P* = .034]; Fig 4, A). However, similar to conventional regulatory T cells, both CCR6⁺ and CCR6^{neg} subtypes from patients with AD proliferated weakly in response to stimulation with anti-CD3 mAb, and this anergy was overcome in the presence of IL-2, although not to the same extent as for subjects without AD (CCR6⁺: 20,220 ± 18,620 cpm vs 38,890 ± 21,090 cpm; CCR6^{neg}: 22,300 ± 20,330 vs 40,280 ± 19,570 cpm [*P* < .05]; Fig 4, A). Interestingly, on activation with anti-CD3 mAb and

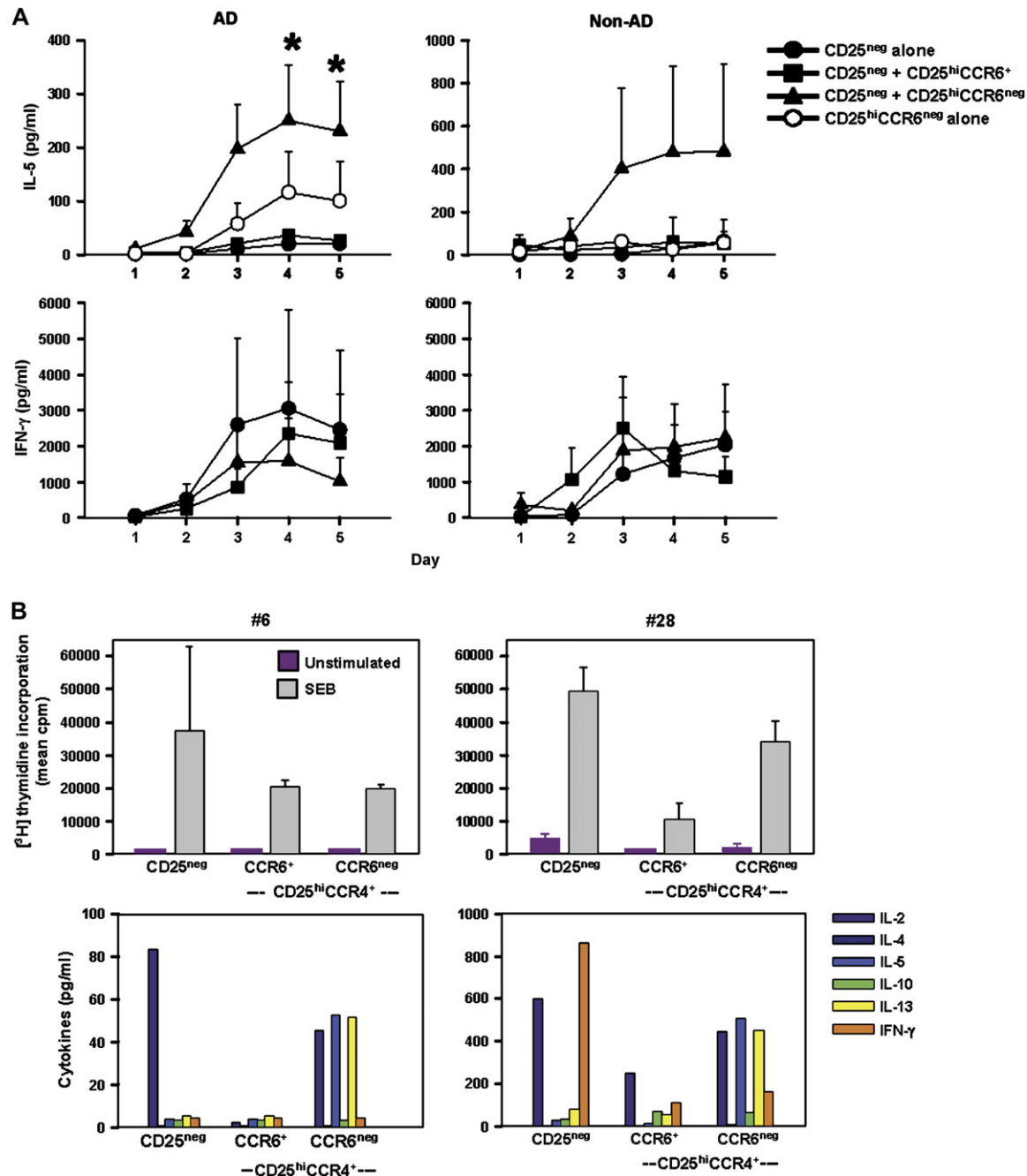


FIG 5. Functional analysis of CD25^{hi} subtypes reveals a T_H2-like population. **A**, CD25^{hi}CCR6⁺ or CD25^{hi}CCR6^{neg} subtypes were cocultured with CD25^{neg}CD4⁺ T cells in the presence of APCs and anti-CD3 mAb and cytokines were measured every 24 hours ($n = 6$ per group). Open symbols, CD25^{hi}CCR6^{neg} cells alone ($n = 4$ per group). * $P < .05$ for CD25^{neg} + CD25^{hi}CCR6^{neg} cells vs CD25^{neg} cells alone. **B**, CD25^{hi} subtypes or CD25^{neg} CD4⁺ T cells were stimulated with SEB and proliferation and cytokines measured on day 3. Error bars represent SDs. cpm, Counts per minute.

IL-2, the CCR6^{neg} subtype secreted higher levels of IL-5 and IL-13 compared with the CCR6⁺ subtype regardless of disease status ($P \leq .036$; Fig 4, B).

In healthy individuals, only CD4⁺ T cells that express the highest levels of CD25 (accounting for 2% to 3% of total CD4⁺ T cells) exhibit the capacity to suppress autologous effector T cells (CD25^{neg}CD4⁺). Although whole CD25^{hi}CD4⁺ T cells from patients with AD suppressed proliferation of effector

T cells, this effect was not observed for either CD25^{hi} subtype (see this article's Fig E2, A and B, in the Online Repository at www.jacionline.org). Surprisingly, IL-5 levels were increased when the CCR6^{neg} subtype from patients with AD was activated in the presence of autologous antigen-presenting cells (APCs), and this effect was further enhanced in the presence of activated effector T cells (Fig 5, A, upper left). By contrast, in subjects without AD, increased IL-5 was observed only when the

CCR6^{neg} subtype was cultured with activated effector T cells, and this was not significant (Fig 5, A, upper right). Production of the T_H1 cytokine IFN- γ was unaltered in the presence of either CD25^{hi} subtype (Fig 5, A, bottom). Thus, activated CD25^{hi} CCR6^{neg} cells secrete T_H2 cytokines and promote IL-5 production in the presence of activated effector T cells.

Bacterial superantigen induces a T_H2-dominated cytokine profile in CD25^{hi}CCR6^{neg} cells

Staphylococcal skin infection may contribute to the pathogenesis of AD through activation of T cells by superantigen. High levels of GITR expression on CD25^{hi} T cells from patients with AD coupled with spontaneous proliferation of CD25^{hi}CCR6^{neg} cells *in vitro* are consistent with *in vivo* activation of these cells in AD, but not in healthy individuals. Thus, we tested whether SEB could induce T_H2 cytokines in CD25^{hi}CCR6^{neg} cells. SEB stimulated strong proliferation of both CD25^{hi} subtypes, as well as CD25^{neg} cells (Fig 5, B, upper panel); however, induction of a T_H2-dominated cytokine profile was specific to the CCR6^{neg} subtype in cultures from both patients with AD and subjects without AD (Fig 5, B, lower panel). Hence, activation and expansion of CD25^{hi}CCR6^{neg} T cells by a clinically relevant antigen drives T_H2 cytokine secretion.

DISCUSSION

We have identified a novel CD25^{hi}CD4⁺ T-cell subtype with T_H2-like properties in patients with AD. This subtype exhibits a phenotype characteristic of CD25^{hi} regulatory T cells (CLA⁺CCR4⁺Foxp3⁺) but lacks expression of the chemokine receptor CCR6. Despite the capacity for whole CD25^{hi}CD4⁺ T cells from patients with AD to suppress autologous effector T cells, the CD25^{hi}CCR6^{neg} T-cell subtype exhibited T_H2-promoting properties as judged by (1) increased production of T_H2 cytokines compared with their CCR6⁺ counterpart on activation, and (2) selective enhancement of IL-5 production in the presence of activated autologous effector T cells. Interestingly, although these features were shared between patients with AD and subjects without AD, the latter effect was significant only in patients with AD. Moreover, although IL-5 levels were increased when CD25^{hi}CCR6^{neg} T cells were activated in the presence of autologous APCs in cultures from patients with AD, this was not the case for the group without AD. Thus, costimulatory molecules or secreted factors expressed by APCs isolated from patients with AD promote secretion of IL-5 by CD25^{hi}CCR6^{neg} T cells. Given that activated effector T cells (ie, CD25^{neg}CD4⁺) secreted only low levels of IL-5 in the absence of CD25^{hi}CCR6^{neg} T cells, the primary source of IL-5 in cultures containing both these cell types was likely CD25^{hi}CCR6^{neg} T cells.

Because CD25^{hi}CCR6^{neg} T cells express skin-homing receptors (CLA and CCR4), it is tempting to speculate that activation and expansion of these cells in the skin in the presence of effector T cells potentiates T_H2-driven inflammatory processes in AD. Consistent with this view, we observed evidence of *in vivo* activation of CD25^{hi} T cells isolated from patients with AD as judged by increased GITR expression and spontaneous proliferation of discrete CD25^{hi} subtypes *ex vivo*. To address how CD25^{hi}CCR6^{neg} T cells may be activated *in vivo*, we tested the responsiveness of these cells to a clinically relevant superantigen (SEB). Sensitization to SEB is common in patients with AD who have elevated

IgE.²⁵ We found that secretion of a T_H2-dominated cytokine profile in response to SEB was restricted to CD25^{hi}CCR6^{neg} T cells. Because SEB induced proliferation of CD25^{hi}CCR6^{neg} T cells, it provides a candidate antigen for driving expansion of these cells *in vivo* in patients with AD. We are currently investigating the T-cell receptor V β repertoire expressed by CD25^{hi} T cells from patients with AD to examine this further.

Despite the suppressive capacity of whole CD25^{hi} T cells in patients with AD, neither CD25^{hi}CCR6⁺ nor CD25^{hi}CCR6^{neg} cells suppressed T-cell proliferation. It is possible that *ex vivo* separation of CCR6⁺ and CCR6^{neg} cells prevents coordinated suppression mediated by these subtypes. This raises the question whether IL-5 production by CCR6^{neg} cells *ex vivo* in the absence of CCR6⁺ cells is relevant to allergic inflammatory processes *in vivo*. It seems unlikely that T_H1 cytokines secreted by CCR6⁺ cells counter-regulate IL-5 produced by CCR6^{neg} cells, because IFN- γ levels were not increased when CCR6⁺ cells were activated in the presence of effector T cells. Nevertheless, further investigation of the interaction between distinct CD25^{hi} subtypes is warranted.

Our findings suggest that expansion of CD25^{hi} T cells is linked to disease severity in patients with AD. Specifically, we have shown that Foxp3⁺ cells with skin-homing potential increase in severe disease, and the frequency of CD25^{hi} T cells is decreased in patients with AD whose skin condition improves after allergen avoidance. Notably, in addition to expression of skin-homing receptors, CD25^{hi} T cells exhibited an effector memory phenotype (CD45RO⁺CCR7^{lo}) characteristic of T cells that migrate preferentially to inflamed tissues.²³ Collectively, these observations support a role for circulating CD25^{hi} T cells in inflammatory processes in the skin. Moreover, our results suggest that allergen exposure, in addition to bacterial superantigen exposure, contributes to expansion of CD25^{hi} T cells in the periphery. Interestingly, CD25^{hi} T-cell subtypes do not proliferate in response to allergen (Reefer and Woodfolk, unpublished data, May 2006). Given their anergic properties, these cells likely require a strong stimulus for activation.²⁶ Thus, allergen may act in concert with other proallergic T-cell-activating factors that are expressed in the skin.

There are 2 distinct scenarios whereby increased circulating T_H2-like CD25^{hi} T cells could contribute to disease severity in AD. One possibility is that these cells are activated and expanded within the skin (or else in regional lymph nodes) by bacterial superantigens or other potent T-cell stimuli that are expressed in AD but not in healthy skin. These cells exert a proinflammatory effect in the skin by virtue of their capacity to traffic to this site and secrete T_H2 cytokines. Consistent with this view, CD25^{hi} T cells from patients with AD exhibited an effector memory phenotype.²³ Alternatively, these cells may arise from skin-resident regulatory T cells which, on receiving specific stimuli, lose expression of CCR6 and leave the skin to join the circulation. This would result in a predominance of effector T cells in the skin that could exacerbate disease. To date, there are conflicting reports on whether putative regulatory T cells (Foxp3⁺CD4⁺) are present in AD skin lesions.^{27,28} Analyzing T cells in AD skin in the context of CCR6 may provide insight into the role of CD25^{hi} subtypes at this site.

In the current study, analysis of Foxp3 expression yielded insight into homeostasis of Foxp3⁺ T cells in patients with AD. Initially, we theorized that changes within the regulatory T-cell compartment underlie aberrant production of IgE in these patients. Such changes could manifest as alterations in the frequency

of CD4⁺ T cells that express Foxp3; however, this was not the case. Although total Foxp3⁺CD4⁺ T cells were increased in patients with AD, the difference was not significant (data not shown). In addition, the distribution of Foxp3⁺ cells among the 5 T-cell subsets that differentially expressed CD25 and CCR4 was remarkably consistent between AD and non-AD groups. Furthermore, the ratio of Foxp3⁺CCR6⁺ and Foxp3⁺CCR6^{neg} T cells within the CD25^{hi} subset was unaltered in AD. It should be noted that expression of CCR6 was decreased on cells that were permeabilized for Foxp3 staining. Thus, the percentage of Foxp3⁺CCR6⁺ cells that were CD25^{hi} was likely underestimated. Nevertheless, our observations point to tight homeostatic control of Foxp3⁺ T cells in the CD4⁺ T-cell pool, as well as in the CD25^{hi} subset.

In summary, we propose that activation and expansion of a novel CD25^{hi}CCR6^{neg} T-cell subtype *in vivo* contributes to the pathogenesis of AD through T_H2-promoting effects. Our findings raise important questions about the role of effector-like regulatory T cells in AD.

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Clinical implications: Expansion and activation of CD25^{hi} T_H2-like cells by bacterial superantigen expressed in atopic dermatitis skin may contribute to disease pathogenesis.

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METHODS

antibodies and reagents

Anti-CD4 (fluorescein isothiocyanate [FITC], phycoerythrin [PE], allophycocyanin-Cyanine-7 [APC-Cy7], PE-Cy7, PE-Cy5), anti-CD25 (APC-Cy7 and PE), anti-CCR4 (Alexa Fluor 647 and PE-Cy7), anti-CD45RO-PE-Cy5, anti-CD69-APC, anti-IL-10R-PE, anti-CCR6-PE-Cy7, and anti-CLA-FITC mAbs were purchased from BD Biosciences (Franklin Lakes, NJ). Anti-CCR6-APC, anti-GITR-FITC, and anti-CCR7-PE mAbs were from R & D Systems (Minneapolis, Minn). Anti-CD4 mAbs (PE-Texas Red and Pacific Blue) were from Caltag (Burlingame, Calif). Anti-TGF β R (R & D Systems) was labeled with Alexa 488 by using a Zenon Human IgG Labeling Kit (Invitrogen, Carlsbad, Calif).

Flow cytometry

For all multicolor analyses, compensation controls (single stains, 1 for each fluorochrome) and gating controls (cells stained with all reagents minus 1) were included.^{E1} Five subsets were selected for detailed phenotypic analyses composed of a mean percentage of total CD4⁺ T cells as follows: (1) CD25^{hi}CCR4⁺: AD = 2.4% $\pm$ 1.4%, non-AD = 0.95% $\pm$ 0.5%; (2) CD25^{mid}CCR4⁺: AD = 3.8% $\pm$ 0.9%, non-AD = 5.7% $\pm$

3.2%; (3) CD25^{neg}CCR4⁺: AD = 6.7% $\pm$ 4.2%, non-AD = 13.7% $\pm$ 4.6%; (4) CD25⁺CCR4^{neg}: AD = 5.8% $\pm$ 4.9%, non-AD = 2.7% $\pm$ 1.6%; (5) CD25^{neg}CCR4^{neg}: AD = 81.3% $\pm$ 6.5%, non-AD = 77.2% $\pm$ 6.2%. In all patients, a fluorescence intensity cutoff >100 was used to delineate the CD25^{hi} subset. Samples were analyzed by using a Cyan ADP benchtop cytometer (DakoCytomation, Fort Collins, Colo) and FlowJo software (Tree Star Inc, Ashland, Ore).

Statistical analysis

All statistical tests were performed by using SPSS 14.0 software (SPSS Inc, Chicago, Ill). An independent Student *t* test was used to analyze between-group cell frequencies and T-cell proliferation studies. Within-group cell frequencies were compared by using a paired 2-sample Student *t* test. For functional T-cell studies, cytokine levels were analyzed by the Wilcoxon signed-rank test. A *P* value of less than .05 was considered significant.

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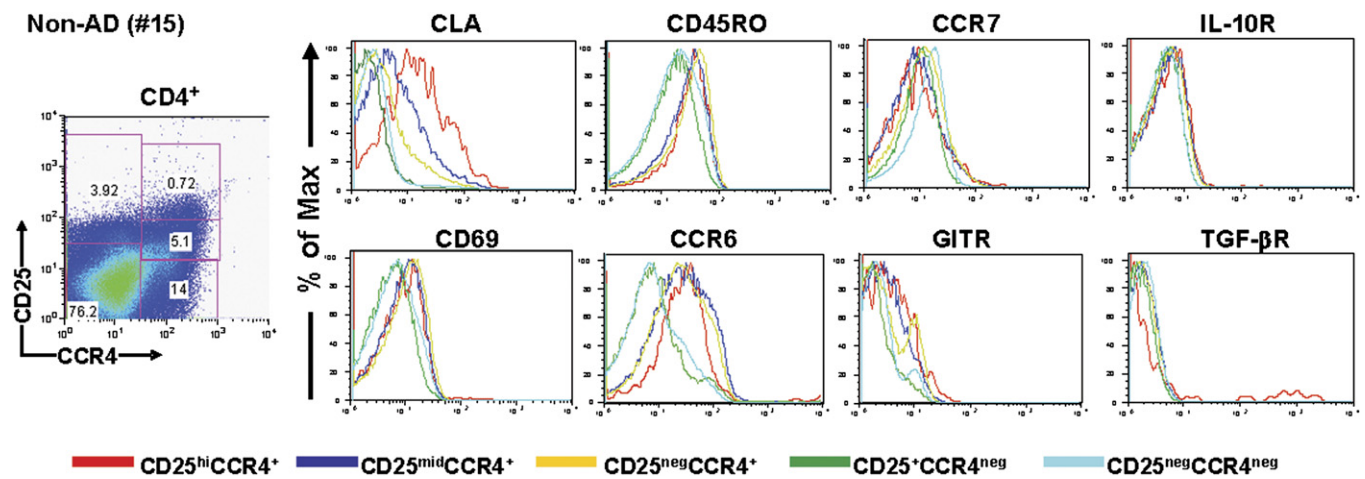


FIG E1. Surface phenotype of CD25^{hi} T cells in an IgE^{low} subject without AD. CD4⁺ T-cell subsets that differentially expressed CD25 and CCR4 were analyzed. Values in *dot plot* indicate cell percentages in each gate (representative data of 6 subjects without AD).

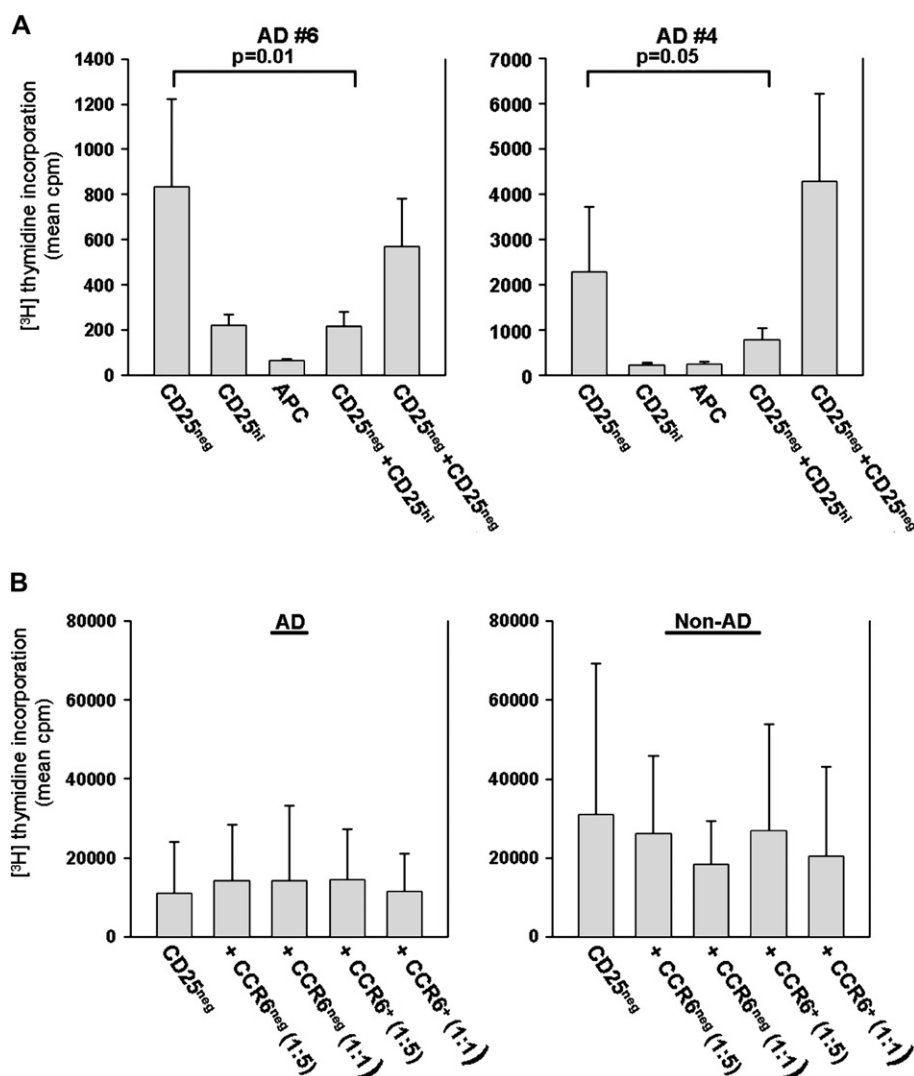


FIG E2. Analysis of the suppressive effect of CD25^{hi} T cells and CD25^{hi} T cell subtypes on T-cell proliferation. **A**, CD25^{neg}CD4⁺ T cells from patients with AD (#4 and #6) were cultured alone or with the same number of CD25^{hi}CD4⁺ T cells or CD25^{neg}CD4⁺ T cells in the presence of irradiated monocytes and plate-bound anti-CD3 mAb. Control wells contained CD25^{hi}CD4⁺ T cells or monocytes (APCs) alone. Proliferation was measured on day 5. Bars represent the means $\pm$ SDs for 6 replicate wells (data are representative of 4 patients with AD). **B**, CD25^{neg}CD4⁺ T cells were cultured alone or with CD25^{hi}CCR6⁺ or CD25^{hi}CCR6^{neg} CD4⁺ T cells at different cell ratios in the presence of irradiated monocytes and plate-bound anti-CD3 mAb (n = 6 per group). Proliferation was measured on day 5. Assays incorporated 3 to 6 replicate wells per condition for each subject. Bars represent the means $\pm$ SDs. cpm, Counts per minute.